Remarks/arguments

Claims 1, 4 - 39 are presently in the case.

Claim Rejections - 35 USC §112

1. Claim 39 stands rejected under 35 U.S.C. 112, first paragraph as failing to comply with the written description requirement. Specifically the claimed invention contains subject matter which allegedly was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s) had possession of the claimed invention at the time the application was filed. The newly added claim recites the limitation that polymerizable co-lipid is not 1,2 Bis[10-(2',4'-hexadienolyoxy)decanoyl]-sn-glycero-d-phosphatidylcholine when the stable liposome-forming lipid is dioleoylphosphatidylethanolamine or dioleoylphosphatidylcholine. The Office Action alleges that there is no support for this limitation.

Applicants respectfully disagree. The Examiner is directed to the examples at page 36, wherein Composition I comprises PEG_{2000}-dioleoylPE, Cholesterol, dioleoylPC and bis $SorbPC_{17,17}$. The colipids in Composition I are not clustered in discrete domains and accordingly do not release their contents at ionizing radiation doses below 200 rads. Furthermore, the Declaration of David O'Brien[1] states in paragraph 14 that the global transition temperature of this liposome would have been substantially below the room temperature. Therefore, this condition would favor random mixing of the lipids and bis-SorbPC where polymerizable colipids are randomly distributed throughout the liposomal

[1] Previously provided by applicants on June 28, 2002.

membrane. Thus Composition I is the composition which does not form discrete domains of colipids and thus there is support for a claim excluding this combination. Clearly there is support in the specification for the claim language. Withdrawal of this rejection is respectfully requested.

Claim Rejections - 35 USC §102(b)

Claims 1, 4, 9-11, 16-17, 23, 32 and 33 stand rejected under 35 U.S.C. 102(b) as being anticipated by Lamparski (Biochemistry, vol. 31., 1992). The Examiner alleges that Lamparski discloses liposomes containing a phospholipid and a polymerizable co-lipid.

The subject matter of the independent Claim 1 and Lamparski are different in many aspects.

Applicants Invention

Generally, in order for liposomes to reach the target site in the patient without significant loss of their contents, passive leakage of the contents must be slow relative to the time required for liposomes to circulate and escape the vasculature. It would be desirable to stimulate enhanced release of the encapsulated agent from the liposomes once the liposomes are at the target site or tumor site in a patient.

Applicants have discovered that by selecting certain lipids and ionizing radiation polymerizable colipids, Applicants can generate a liposome in which the colipids in the liposome are clustered in discrete domains when the unpolymerized liposome is administered to a patient. Applicants have also shown that if the colipids are clustered in discrete domains at the time of ionizing radiation, the ionizing radiation has a greater effect in destabilizing the liposome, thereby releasing more of the agents present within the liposome. Accordingly, it is

possible to achieve enhanced release of the encapsulated agent from the liposomes once the liposomes are at the target site. Use of low doses of ionizing radiation is beneficial, because it can penetrate the patient's body and be directed to the site where release of the agent is desired.

The Case Law

MPEP §2131 provides, "'A claim is anticipated only if *each* and *every* element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference.[2],. A showing that the prior art reference lack[ed] the characteristics of the claimed invention would in fact negate the assertion that the claimed invention was described in the prior art.[3].

Analysis

Applicants respectfully point out that the global transition temperature (T_M) of the liposomes disclosed in Lamparski are substantially below the room temperature. Accordingly, the temperature at which the liposomes disclosed in Lamparski would potentially form discrete domains would be at a temperature which is below room temperature.

The Declaration of Dr. O'Brien[4] states that in Lamparski the liposomes were composed of DOPE or DOPC in either a 2:1 or 3:1 molar ratio with bis-SorbPC (para. 6).

> "Therefore, the liposomes prepared and studied in Lamparski were designed to form fluid phase liposomes at room temperature and above. Furthermore, at 3:1 molar ratio with bis-SorbPC, the global transition temperature would have been substantially below the room temperature. These conditions favor random mixing of the lipids and bis-SorbPC where polymerizable colipids are randomly distributed throughout the liposomal membrane."

[2] *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051-1053 (Fed. Cir. 1987)
[3] *In re Mills, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990)*
[4] The Declaration of Dr. O'Brien was previously filed in this application on June 28, 2002.

First Applicants note that David O'Brien is one of the authors of the Lamparski article. Secondly, the liposomes disclosed in Lamparski would not form discrete domains in a patient because (as indicated in Dr. O'Brien's declaration) body temperature (37°C) is above the temperature at which the liposomes of Lamparski form discrete domains. Accordingly, the liposomes taught by Lamparski do not anticipate the claimed invention. In this case, there is no disclosure, either inherent or explicit, in Lamparski which would indicate *the colipids are clustered in discrete domains after the liposome is administered to a patient.* Accordingly, Lamparski et al. does not disclose *each* and *every* element as set forth in the claims, either expressly or inherently. The liposomes taught by Lamparski do not anticipate the claimed invention.

The Examiner improperly dismisses the Declaration of David O'Brien wherein he argues that the main transition temperature of DOPC and DOPE (used by Lamparski) are -20C and -10C degrees respectively and that the main phase transition temperature of the bis-sorbPC used is 27C degrees. The Office action also refers to the statement that at a 3:1 molar ratio with bis-sorbPC, the global transition temperature would have been substantially below the room temperature. The Office Action indicates that these arguments are deemed *speculative* since the reference of Bennett and O'Brien (Biochemistry vol. 34, 1995) already of record indicates to be otherwise. The Examiner indicates that Bennett et al. allegedly finds the global transition temperature of the same mixture (DOPE/bis-sorb PC) to be 49C degrees, which is well above the room temperature. The Examiner also notes that newly added claim 39 recites the same DOPC and DOPE.

This rejection by the Patent Office is clearly erroneous for a number of reasons. First, David O'Brien is an author of both the Lamparski *et al* article and the Bennett and O'Brien article. Clearly the statements of David O'Brien are not "*speculative*" since David O'Brien is speaking about experiments on which he collaborated.

Secondly, this rejection is erroneous because it is factually wrong. As set forth in the enclosed Declaration by Bruce Bondurant and, as would be known by one skilled in the art of liposomes, the T_I described in Table 1 of the Bennett and O'Brien article is the temperature at the lower end of the temperature range ΔT_I that is defined by Ellens *et al.,* to be the temperature range over which a fluid lamellar phase of the liposome forms inter-lamellar assemblies that are an intermediate step to the inverted cubic phase, which will, at yet higher temperatures form the inverted H_{II} phase at a transition temperature called T_H. This is a completely different temperature from the main phase transition temperature (T_M) discussed in the current application. The main phase transition temperature (T_M) is the temperature at which the liposome lipid bilayer changes from a gel to liquid crystalline phase. The T_I is not the same as the T_M.

The case law has clearly established that in considering affidavit evidence, the Examiner must consider all of the evidence of record anew.[5] "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument" [6] Furthermore, the Federal Court of Appeals held in *In re Alton*, "[w]e are aware of no reason why opinion evidence relating to a fact issue should not be considered by an

[5] *In re Rinehart 531* F.2d 1084, 189 USPQ 143 (CCPA 1976) and *In re Piasecki* 745 F 2d. 1015, 226 USPQ 881 (Fed. Cir. 1985)

examiner"[7]. Applicants also respectfully draw the Examiner's attention to the Utility Examination Guidelines[8] which states that, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a disagreement over the significance or meaning of the facts offered".

Therefore Claim 1 is not anticipated by Lamparski et al. and the Examiner is respectfully requested to reconsider and withdraw the rejection.

Claim Rejections - 35 USC §103(a)

1. Claims 1, 4-5, 9-11, and 16-31-33, 36 and 38-39 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Lamparski in view of either Heldebrant (U.S. Patent No. 5,061,484) or Charych (U.S. Patent No. 6,180,135) in further combination with Hallahan (U.S. Patent No. 6,159,443). It allegedly would have been obvious to use the liposomes of Lamparski for the delivery of the diagnostic or therapeutic agents with a reasonable expectation of success since Lamparski provides guidance as to how to prepare the liposomes and suggests their use.

The Office Action alleges that Lamparski teaches the instant liposomes and the destabilizing effect of ultra-violet light on the liposomal membrane. "What is lacking in Lamparski are the teachings of the use of X-rays for polymerization of the lipids and destabilizing the liposome. Although Lamparski does not specifically teach a method of administration of a therapeutic agent or a diagnostic agent, Lamparski allegedly suggests the

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[6] *In re Alton* 37 USPQ2d 1578 (Fed. Cir 1966) at 1584 quoting *In re Oetiker* 977 F 2d at 1445, u2 USPQ2d at 1444.

[7] *In re Alton*, supra

applicability of the radiation induced destabilizing of the liposome and the regulation of the release of the biological agents (page 693)."

The Office Action states that Heldebrant allegedly discloses the administration of liposomal composition to tumor bearing mice and teaches that liposomal lipids can be polymerized by either UV radiation or by x-rays.

The Office Action states that Charych allegedly teaches that liposomal lipids can be polymerized by either UV radiation or by X-rays.

The Office Action states that Hallahan allegedly discloses X-ray guided drug delivery to treat various neoplasms.

The Office Action agrees that Lamparski teaches only the application of ultraviolet light as the source for polymerization. However, in the absence of a showing of criticality, the Patent Office deems it obvious to use any form of ionization as long as they polymerize the lipid. The use of X-rays as the ionizing radiation with the liposomes of Lamparski would allegedly have been obvious to one of ordinary skill in the art since X-rays are not only another form of ionizing radiation to polymerize the liposomal lipids as shown by Heldebrant or Charych, but also provide an improved method of delivery when combined with delivery vehicles such as the liposomes shown by Hallahan. Hallahan discloses administering a therapeutic agent or diagnostic agent in a delivery vehicle (liposome) . The liposome also contain antibodies attached to them.

Applicants respectfully disagree.

The Case Law

[8] Part IIB, 66 Fed. Reg. 1098 (2001)

For the claimed subject matter to be obvious in view of a combination of prior art references, the prior art must suggest the combination to one of ordinary skill in the art and reveal that one of such skill would have a reasonable expectation of success in carrying out the invention[9].

It is impermissible for the Examiner simply to use hindsight reconstruction of the claimed invention, using the applicant's structure as a template and selecting elements from the references to fill the gaps.[10] There must be a teaching or suggestion in the art to combine the references and a reasonable expectation of success.

Lamparski has already been discussed in the rejection under 35 U.S.C. §102. First, Lamparski does not teach that the colipids should be clustered in discrete domains at the time of polymerization in order to further destabilize the liposomes. Secondly, as the Examiner agreed at page 4, Lamparski does not teach or suggest the use of ionizing radiation to destabilize the liposome. Finally Lamparski does not teach or suggest the liposomes of the claimed invention.

It is incorrect for the Office Action to state on pages 4-5 that Lamparski suggests the applicability of the radiation induced destabilizing of the liposome and the regulation of the release of the biological agents. In this statement the Patent Office is contradicting itself. The Examiner agrees that Lamparski teaches the use of UV light and not the use of ionizing radiation. As one of skill in the art would know, and as set forth in the Declaration by Bruce Bondurant, Ph.D., UV light polymerizes bis-SorbPC$_{17, 17}$ through photo-addition. Ionizing

[9] *In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).*
[10] *In re Gorman* 933 F2d. 982, 18 USPQ2d 1885 (Fed. Cir. 1991)

radiation induced polymerization of bis-SorbPC$_{17, 17}$ occurs through radical chain reaction. These chemical reactions are not the same.

Charych teaches polymerized polymeric assemblies (ie. liposomes) which change from a blue to red color when exposed to an analyte as a diagnostic product. In Charych, diacetylene monomers are combined into a liposome and the liposome is polymerized with a UV lamp in vitro. Charych does teach that X-rays are another polymerization means. Charych does not teach the administration of his liposomes to patients. Charych does not teach a mixture of lipids and colipids. Charych does not teach or suggest the benefit of having the colipid form discrete domains before polymerization. Charych does not teach or suggest administration of unpolymerized liposomes to a patient. Charych does not teach or suggest selecting a colipid and lipid such that the colipid would form discrete domains in the unpolymerized liposome when administered to a patient.

Heldebrant teaches a stable perfluorochemical emulsion which comprises perfluorochemical particles in stabilized vesicles. The stabilized vesicles comprise a biocompatible polymer formed by coating the perfluorochemical particles with one or more phospholipid monomers and polymerizing the monomers. The stabilized/polymerized liposome is then administered to a patient. The purpose of the polymerization is to generate more stable emulsions which (1) can withstand higher and longer sterilization temperatures and times, (2) possess greater stability after sterilization with permits longer storage times; and (3) have longer circulating in vivo half-lives. Heldebrant does not teach or suggest selecting a colipid and lipid such that the colipid would form discrete domains in the liposome when administered to a patient. Heldebrant does not teach or suggest administering an unpolymerized liposome to a patient. Heldebrant does not teach or suggest releasing the

perfluorochemical from the liposome. In fact, Heldebrant specifically teaches away from the claimed invention by indicating that the liposome should be composed of only polymerizable colipids and be completely polymerized before administration to a patient to ensure stability of the polymerized liposome in the patient and to prevent the release of the perfluorochemical (Col. 6, lines 29 - 40).

Hallahan teaches a targeting technique of delivering an active agent to a target tissue, particularly neoplastic tissue. Hallahan teaches the use of ionizing radiation targeted at tumor sites to induce platelet aggregation. Hallahan teaches the attachment of a delivery vehicles to platelets to increase the targeting of the delivery vehicles. One of many different targeting vehicles mentioned by Hallahan are liposomes. Hallahan does not teach or suggest the composition of the liposomes except to indicate that that the liposomes require conjugation to a peptide or antibody that preferentially binds activated platelets. Hallahan does not teach or suggest the use of X-rays to polymerize any type of unpolymerized liposome in the patient. Accordingly, Hallahan does not teach or suggest a liposome comprising a lipid and an ionizing radiation polymerizable colipid which forms discrete domains when administered to a patient.

Accordingly, none of the references, either alone or in combination, teach or suggest a liposome delivery system comprising a lipid and ionizing radiation polymerizable colipid which forms discrete domains when administered to a patient. Contrary to the statements on page 5 of the Office Action it would not have been obvious to use the liposomes of Lamparski for the delivery of diagnostic or therapeutic agents with a reasonable expectation of success. First, Lamparski does not provide guidance on how to prepare the claimed liposomes or suggest their use as a therapeutic or diagnostic agent. The Examiner is requested to point to

the section of Lamparski where such guidance and teaching is provided. Secondly, while Charych does teach the use of X-rays for the liposomes taught by Charych, , Charych does not teach the liposomes of the invention or the use of X-rays for the polymerization of the liposomes of the invention. Further, Charych does not teach the administration of such liposomes to patients for therapeutic or diagnostic purposes. None of the references teach or suggest, either alone or in combination, the selection of lipids and colipids to form a unpolymerized liposome delivery system which forms discrete domains after administration to a patient. Third, Heldebrant specifically teaches away from the claimed invention by indicating that the liposome should be composed of only polymerizable colipids and be completely polymerized before administration to a patient. Finally, Hallahan does not teach anything about the liposome composition except that it should be conjugated to a peptide or antibody which binds platelets. Accordingly, a combination of the cited references would not result in the claimed invention, much less teach or suggest the claimed invention or a reasonable expectation of success.

In addition, none of the references teach or suggest that ionizing radiation energies that are much higher than UV would cause the clustered colipids to polymerize thereby releasing the contents from the liposome. UV radiation excites an electron. On the other hand , ionizing radiation photons in their interaction with matter can result in the complete displacement of an electron. Accordingly, it is not obvious that the claimed liposome delivery system would comprise an ionizing radiation sensitive colipid. Absent such a teaching the claimed invention is not obvious and withdrawal of this rejection is respectfully requested.

The Office Action does not specifically address claims 20, 24 or 27 which are directed to the method claims in this rejection. Absent reasons for the rejection, the Examiner has

failed to meet his burden of proving an *prima facie* case of obviousness. Furthermore, for the reasons set forth above, the references alone or together do not teach or suggest the methods of claims 20, 24, or 27.

Withdrawal of this rejection is respectfully requested.

2. Claims 5-8 and 12-15, and 34-35 and 37 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Lamparski in view of either Heldebrant ('484 patent"), Charych (the '135 patent") in further combination with Hallahan ("the '443 patent") and further in view of Woodle (BB 1992). The Examiner indicates that what is lacking in Lamparski and the other references already discussed are the teaching of the inclusion of PEG in the liposomal compositions.

This rejection is also respectfully traversed. The teachings of Lamparski, Heldebrant, Charych and Hallahan have already been discussed.

Woodle does teach the use of pegylated lipids in liposomes. Woodle does not teach the selection of lipids and ionizing radiation polymerizable colipids such that the colipids in the unpolymerized liposome form discrete domains. Absent such a teaching, Woodle does not cure the deficiencies of the other references and the claims are not rendered obvious.

Consequently, there exists no intrinsic basis or extrinsic justification for the proposed combination of Lamparski with Heldebrant, Charych, Hallahan and Woodle and *prima facie* obviousness has not been established. Applicants respectfully traverse the rejection of those claims.

Conclusion

Reconsideration, and allowance of claims 1 and 4-39 are therefore respectfully requested. Should there be any further issues outstanding, the Examiner is invited to contact the undersigned attorney at the telephone number shown below.

Please charge any fees, including any fees for extension of time, or credit overpayment to Deposit Account No. 08-1641 (referencing Attorney's Docket No. 15907-0022).

Respectfully submitted,

By: Leslie Mooi
Leslie A. Mooi
Registration No. 37,047

Date: April 14, 2005

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PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of O'Brien et al.	Examiner: Gollamudi S. Kishore, Ph.D.
For: **RADIATION SENSITIVE LIPOSOMES**	Group Art Unit: 1615
Serial No.: 09/728,716	
Filed: November 30, 2000	

DECLARATION PURSUANT TO 37 CFR §1.132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Bruce Bondurant, Ph.D., hereby declare as follows:

1. I am a Research Associate at the Duke University Medical Center in Durham, North Carolina and co-inventor of the invention disclosed and claimed in the above-referenced patent application, filed November 30, 2000. I consider myself to be skilled in the art of organic and polymer chemistry. My *curriculum vitae* is appended hereto (Exhibit A).

2. I am familiar with the specification and claims in the above-referenced patent application. I have reviewed the amended claims which require the polymerizable colipids to be in the form of discrete domains after administration to a patient.

3. I am familiar with the Declaration of David F. O'Brien, dated June 27, 2002 and I agree with statements made with one exception. In paragraph 10 of David F. O'Brien's Declaration, the main phase transition temperature of the bis-SorbPC used in

the Lamparski study should be 29°C, as was stated in paragraph 11, Table 1 of David O'Brien's Declaration for bis-SorbPC$_{17,17}$.

4. I have read the article by Lamparski *et al.* titled "Photoinduced Destabilization of Liposomes", *Biochemistry*, vol. 31(3):685-694 (1992) and I am familiar with the subject matter of the teachings of Lamparski *et al.* (1992).

5. I have read the article by Bennett and O'Brien titled "Photoactivated Enhancement of Liposome Fusion", *Biochemistry*, vol. 34(9):3102-3113 (1995) and I am familiar with the subject matter of the teachings of Bennett and O'Brien.

6. I have read the article by Ellens *et al.* titled "Membrane Fusion and Inverted Phases", *Biochemistry*, vol. 28(9):3692-3703 (1989) appended hereto (Exhibit B) which is cited in the article by Bennett and O'Brien and I am familiar with the subject matter of the teachings of Ellens *et al.*

7. I have read the article by Lamparski *et al.* titled "Thermotropic Properties of Model Membranes Composed of Polymerizable Lipids. 1. Phosphatidylcholines Containing Terminal Acryloyl, Methacryoyl and Sorbyl Groups", *J. Am. Chem. Soc.*, vol. 115:8096-8102 (1993) appended hereto (Exhibit C) and I am familiar with the subject matter of the teachings of Lamparski *et al.*(1993).

8. Lipid assemblies are polymorphic in that they will take on different thermodynamic structures depending on the nature of the lipid molecule and on environmental considerations such as pH, temperature, and solutes that are present.

9. Bennett and O'Brien in Table 1 on page 3105 refer to the T_I (onset) and T_H (peak). T_I is the temperature at the lower end of the temperature range ΔT_I that is defined by Ellens *et al.* (Exhibit B) to be the temperature range over which a fluid lamellar phase of the liposome forms inter-lamellar assemblies that are an intermediate step to the inverted cubic phase, which will, at yet higher temperatures form the inverted H_{II} phase

at a transition temperature called T_H, listed as a second column on Table 1 of 3105 in the Bennett reference. That is why T_I is described in parenthesis as "(onset)". It marks the onset of the formation of non-lamellar phases.

10. Lamparski *et al.* (1993) (Exhibit C) define the main phase transition temperature (T_m) to be the temperature at which the lipid bilayer changes from a gel to liquid crystalline phase. Hydrated bilayers of a pure lipid exist in a solid-like or gel like lamellar state when the experimental temperature is below the main phase transition temperature or global transition temperature (T_m) of the lipid. Mixtures of lipids are also in a solid-like or gel like lamellar state when the temperature is near or below the T_m of the lowest melting lipid in the mixture.

11. The main phase transition temperature (T_m) of a lipid and the temperature at which a fluid lamellar phase of the liposome begins to form inter-lamellar assemblies (T_I) are different temperatures.

12. The liposomes prepared and studied in Lamparski *et al.* (1992) were designed to form fluid phase liposomes at room temperature and above. At a 3:1 molar ratio of dioleoylphosphatidylethanolamine ("DOPE") or dioleoylphosphatidylcholine ("DOPC") with 1,2-bis-[10-[(2',4'-hexadienoyloxy)decanoyl]-*sn*-glycero-3-phosphatidylcholine ("bis-SorbPC$_{17,17}$"), the global transition temperature (T_m) would have been substantially below room temperature. These conditions favor random mixing of the lipids and bis-SorbPC$_{17,17}$ where polymerizable colipids are randomly distributed throughout the liposomal membrane.

13. The currently described invention of radiation-sensitive liposomes involves the polymerization of pre-existing, thermodynamically separated, gel phase domains of polymerizable lipids that coexist with gel phase domains of non-polymerizable lipids. The polymerization of these domains does not produce non-lamellar phases, but rather disrupts the chain packing in the gel phase domain, in a manner similar to what heating

the lipid mixture over the main phase transition would do. This results in an increase in the permeability of these domains.

14. To claim that the radiation induced polymerization and destabilization of the radiation sensitive liposomes described in the above-referenced patent application follows logically from the photo-polymerization of DOPE/ bis-SorbPC$_{17,17}$ liposomes is incorrect. The photopolymerization of bis-SorbPC$_{17,17}$ occurs through the photo-addition of an excited state molecule without radical intermediates. The ionizing radiation induced polymerization of bis-SorbPC$_{17,17}$ occurs through a radical chain reaction. The two mechanisms are entirely different, and the success of one reaction does not necessarily imply that the other will occur.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the above identified application or any patent issued thereon.

So declared

Bruce Bondurant 4/13/05

Bruce Bondurant, Ph.D. Date

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QUALIFICATIONS

Organic chemist with good problem solving skills and significant understanding of spectroscopic and calorimetric analytical techniques and biochemistry

EXPERIENCE

Research Associate — 2004-present
Duke University Medical Center, Durham, North Carolina
- Designed and manufactured radiation sensitive liposomes for drug delivery
- Invented and synthesized Radio-sensitizing amphiphiles
- Synthesized high purity polymerizable lipids

Adjunct Faculty — 2003-2004
Albuquerque Technical Vocational Institute, Albuquerque, New Mexico
- Instructed introductory chemistry students in laboratory technique and chemistry fundamentals
- Evaluated student performance

Post Doctoral Appointee — 2000 -2003
Sandia National Laboratories, Albuquerque, New Mexico
- Designed and synthesized novel, functionalized, fluorescent lipids for protein binding experiments
- Measured protein binding to functionalized liposomes by fluorescence spectroscopy and isothermal titration calorimetry

Research Assistant — 1995 - 2000
David F. O'Brien Research Group, University of Arizona, Dept. of Chemistry, Tucson, Arizona
- Designed and synthesized polymerizable and polymer-grafted lipid molecules for research on the biophysical chemistry of membranes
- Developed and implemented reverse phase preparative HPLC separations techniques for polymer grafted lipids and polymerizable lipids
- Performed spectroscopic and biophysical experiments including fluorescence spectroscopy, QELS, and differential scanning calorimetry on lipid membranes and colloidal particles
- Managed safety and hazardous materials concerns for a synthetic organic research laboratory

Teaching Assistant — 1993 – 1995
University of Arizona, Dept. of Chemistry, Tucson, Arizona
- Taught laboratory technique to students of organic chemistry
- Conducted chemistry outreach demonstrations in schools

Research and Production Technician — 1989 -1993
Molecular Probes Inc., Eugene, Oregon
- Developed synthesis and chromatographic separations techniques for organic fluorescent dyes and their synthetic intermediates
- Implemented scale up techniques from laboratory to pilot plant scale production of a cholesterol test indicator molecule

EDUCATION

BA, Chemistry: University of Oregon — 1986
Ph.D. Organic Chemistry, minor in Biochemistry
University of Arizona — 2000

TRAINING

Radiation Safety — 2004
Advanced Pressure Safety — 2003
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SELECTED PUBLICATIONS

Polymerization of Supramolecular Assemblies: Comparison of Lamellar and Inverted Hexagonal Phases *Tetrahedron* Srisri, Warunee; Lee, Youn-Sik; Sisson, Thomas M.; Bondurant, Bruce; O'Brien, David F. Vol.53, No45, pp.15397-15414, 1997

Liposome-Cell Interactions in Vitro: Effect of Liposome Surface Charge on the Binding and Endocytosis of Conventional and Sterically Stabilized Liposomes . Miller, Christina R; Bondurant, Bruce; McLean, Shannon D.; McGovern, Kathy A.; O'Brien, David F. *Biochemistry* (1998), 37(37), 12875-12883

Photoinduced Destabilization of Sterically Stabilized Liposomes . Bondurant, Bruce; Obrien, David F. *JACS* (1998), 120(51), 13541-13542

Measurements of interbilayer Forces and Protein Adsorption on Uncharged Lipid Bilayers Exposing Terminally Grafted PEG chains. Efremova, Nadezhda V.; Bondurant, Bruce; O'Brien, David F.; Leckband, Deborah E. *Biochemistry* (2000), 39(12), 3441 - 3451

Visible Light-Stimulated Destabilization of PEG-Liposomes. Mueler, Anja; Bondurant, Bruce; Obrien, David F. *Macromolecules* 2000; 33(13); 4799-4804.

Formation of Self Assembled air Stable Lipid Bilayer Membranes on Solid Supports. Ross, Eric E.; Bondurant, Bruce; Spratt, Tony; Conboy, John C.; O'Brien, David F.; Saavedra, S. Scott. Langmuir (2001), 17(18), 2305-2307

Photoinitiated Destabilization of Sterically Stabilized Liposomes. Bondurant, Bruce; Mueller, Anja; O'Brien, David F. *Biochimica et Biophysica acta* (2001), 1511(1), 113-112

Radiation-sensitive Liposomes Containing Polymerizable Lipids for Drug Delivery. O'Brien, David F.; McGovern, Kathy A.; Bondurant, Bruce; Sutherland, Robert M. PCT Int. appl. (2001) US 1999-168100P

Stabilized Biocompatible, Supported Lipid Membranes. Saavedra, Scott S.; O'Brien, David F.; Ross, Eric E.; Bondurant, Bruce; Conboy, John C. (USA) PCT Int. Appl. (2002)

Optical Scanning Probe Analysis of Glycolipid Reorganization upon Concanavalin A Binding to Mannose-Coated Lipid Bilayers. Bondurant, Bruce; Last, Julie A.; Waggoner, Tina A.; Slade, Andrea; Sasaki, Darryl Y. *Langmuir* (2003), 19 (5), 1829 - 1837

Membrane Fusion and Inverted Phases†

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Received April 14, 1988; Revised Manuscript Received January 12, 1989

ABSTRACT: We have found a correlation between liposome fusion kinetics and lipid phase behavior for several inverted phase forming lipids. *N*-Methylated dioleoylphosphatidylethanolamine (DOPE-Me), or mixtures of dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC), will form an inverted hexagonal phase (H_{II}) at high temperatures (above T_H), a lamellar phase (L_α) at low temperatures, and an isotropic/inverted cubic phase at intermediate temperatures, which is defined by the appearance of narrow isotropic ^{31}P NMR resonances. The phase behavior has been verified by using high-sensitivity DSC, ^{31}P NMR, freeze–fracture electron microscopy, and X-ray diffraction. The temperature range over which the narrow isotropic resonances occur is defined as ΔT_I, and the range ends at T_H. Extruded liposomes (~0.2 μm in diameter) composed of these lipids show fusion and leakage kinetics which are strongly correlated with the temperatures of these phase transitions. At temperatures below ΔT_I, where the lipid phase is L_α, there is little or no fusion, i.e., mixing of aqueous contents, or leakage. However, as the temperature reaches ΔT_I, there is a rapid increase in both fusion and leakage rates. At temperatures above T_H, the liposomes show aggregation-dependent lysis, as the rapid formation of H_{II} phase precursors disrupts the membranes. We show that the correspondence between the fusion and leakage kinetics and the observed phase behavior is easily rationalized in terms of a recent kinetic theory of L_α/inverted phase transitions. In particular, it is likely that membrane fusion and the L_α/inverted cubic phase transition proceed via a common set of intermembrane intermediates.

Membrane fusion can be analyzed at two levels: (i) kinetic—to ascertain which step of the overall process is rate limiting and what factors influence that step; (ii) structural—to identify the intermembrane structures which either cause fusion or evolve from the fusion intermediate (Bentz & Ellens, 1988). The kinetic analysis of vesicle fusion is now well developed (Nir et al., 1980; Bentz et al., 1983, 1985a, 1988; Ellens et al., 1984). Fluorometric assays for contents mixing, leakage, and the mixing of membrane components have greatly facilitated this work (Düzgüneş & Bentz, 1988).

The structural analysis of liposome fusion began with studies of lipid phase behavior. It was postulated that the intermediates of liposome fusion formed during the course of lipid phase transitions (Suurkuusk et al., 1976; Papahadjopoulos et al., 1977; Verkleij et al., 1980). Subsequent studies found that the observed temperature dependence of liposome fusion did not correlate particularly well with the phase transition tempertures (Düzgüneş et al., 1984; Bentz et al., 1985a,b; Ellens et al., 1986a; Lentz et al., 1987).

There has been some success with correlating liposome fusion kinetics with lateral phase segregation of the lipid constituents (Düzgüneş et al., 1984; Silvius & Gagné, 1984a,b; Leventis et al., 1986; Bentz et al., 1987). This area of study is an important special case of the general problem in that it permits us to understand whether or not the "heterogeneous" membrane might produce local domains of lipids which are fusion competent. However, it does not explain anything more about the fusion intermediate itself than could be determined by using membranes composed of the lipids in the fusion-competent domains. The exception to this would arise if fusion is due to the boundaries between the segregated domains.

Using *N*-methyldioleoylphosphatidylethanolamine (DOPE-Me),[1] Ellens et al. (1986b) found enhanced liposome fusion kinetics in the temperature range in which Gagné et al. (1985) observed isotropic ^{31}P NMR resonances in bulk dispersions

† This investigation was supported by Research Grant NIH-GM-31506 and UCSF Academic Senate Grant (J.B.), a senior postdoctoral grant from the California Branch of the American Cancer Society (H.E.), Grant NIH-HL-23853 (P.L.Y.), and Grant GR/E 33769 from the Science and Engineering Research Council (U.K.) (P.J.Q.).

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[1] Abbreviations: LUV, large unilamellar vesicle(s), ~0.1–0.2-μm diameter; MLV, multilamellar vesicles; SUV, small and sonicated vesicle(s), <0.05-μm diameter; CL, cardiolipin; PA, phosphatidate; PC, phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, N-methylated DOPE; EPE, egg phosphatidylethanolamine; TPE, phosphatidylethanolamine prepared from egg phosphatidylcholine by transesterification; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine prepared from TPE; PS, phosphatidylserine; CHEMS, cholesteryl hemisuccinate; ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); DPA, dipicolinic acid; Tb, terbium Tb^{3+}; T_c, temperature of the lamellar (gel) L_β–(liquid-crystalline) L_α phase transition; ΔT_I, temperature range from the onset of isotropic ^{31}P NMR resonances to the T_H determined by DSC; T_H, temperature of the L_α-inverted hexagonal H_{II} phase transition; IMI, inverted micelle intermediate; ILA, interlamellar attachment.

0006-2960/89/0428-3692$01.50/0

of the same lipid. Because the proper phase of the lipid under these conditions is not known, we refer to the lipid as being in the *isotropic state* at those temperatures where the isotropic resonances are found.[2] When the temperature exceeds the L_α/H_{II} phase transition temperature, T_H, these liposomes undergo a contact-mediated lysis; i.e., the mixing of aqueous contents associated with fusion is abolished, as has been found for many other systems (Bentz et al., 1985b, 1987; Ellens et al., 1986a,b). Above T_H, there is also a prodigious amount of lipid mixing as the liposomal membranes are transformed to H_{II} phase precursors and the bilayers cease to exist.

We would like to understand the intermembrane structures which produce these isotropic resonances, since they are putative fusion structures. We know that the isotropic resonances are due to a fraction of the phospholipids in the system experiencing isotropic motional averaging on the ^{31}P NMR time scale [ca. 20 μs (Campbell et al., 1979; Larsen et al., 1987)]. They appear under the same circumstances as lipidic particle morphology, observed by electron microscopy [e.g., see de Kruijff et al. (1979), Gagnē et al. (1985), Boni and Hui (1983), and Hui et al. (1983)], and as amorphous and inverted cubic phases, observed by X-ray diffraction [e.g., see Rilfors et al. (1982, 1986), Boni and Hui (1983), Hui et al. (1983), Kirk (1984), and Gruner et al. (1988)]. However, there is no definitive answer as to the structure of these intermediates (Hui et al., 1981, 1983; Boni & Hui, 1983; Verkleij, 1984; Borovagin et al., 1982; Cullis et al., 1985).

Recent theoretical studies on the mechanism of L_α/H_{II} phase transitions postulated a sequence of intermembrane structures which can account for most of the NMR, DSC, and morphological data concerning these transitions (Siegel, 1986a–c, 1987; Siegel et al., 1988). The postulated intermediates are consistent with models for the relative chemical potentials of inverted phase structures (Kirk et al., 1984; Gruner, 1985, 1986), as well as with results of X-ray diffraction studies of these systems (Caffrey, 1985, 1987; Tate & Gruner, 1987; Gruner et al., 1988). These theoretical studies also predict that a subset of systems (including DOPE-Me and PE/PC mixtures) with L_α/H_{II} transitions should form a type of intermediate that would produce isotropic ^{31}P NMR resonances in a temperature interval starting tens of degrees below T_H (Siegel, 1986c).

Here we have studied the fusion and leakage of liposomes composed of DOPE-Me and of several DOPE/DOPC mixtures, as well as the equilibrium phase behavior of these systems using high-sensitivity DSC, ^{31}P NMR, time-resolved X-ray diffraction, and rapid freeze–fracture electron microscopy. We conclude that liposome fusion, isotropic ^{31}P NMR resonances, and inverted cubic lattice formation share a common molecular intermediate. Of course, this mechanism of fusion is relevant only in lipid systems that have a nearby H_{II} phase boundary, and then only if isotropic ^{31}P NMR resonances are observed on the first heating scan (Ellens et al., 1986b; Siegel, 1986b,c; Siegel et al., 1988). The current status of other liposome fusion mechanisms has been discussed elsewhere (Bentz & Ellens, 1988; Bentz et al., 1988).

Materials and Methods

Dioleoylphosphatidylethanolamine (DOPE), DOPE with a single methyl substitution at the amino group (DOPE-Me), dioleoylphosphatidylcholine (DOPC), and *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE prepared from transesterified egg PC (NBD-PE) were purchased from Avanti Polar lipids (Birmingham, AL). 1-Aminonaphthalene-3,6,8-trisulfonic acid (disodium salt) (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes Inc. (Junction City, OR). Terbium chloride ($TbCl_3$) was from Aesar Rare Earth Products (Seabrook, NH), nitrilotriacetic acid (NTA) was from Aldrich (Milwaukee, WI), and dipicolinic acid (DPA) was from Sigma.

Liposome Preparation. The liposomes were prepared according to Szoka and Papahadjopoulos (1978). Further details on all these procedures can be found in Ellens et al. (1985, 1986a,b) and Bentz et al. (1988). For the ANTS/DPX leakage and fusion assay (Ellens et al., 1985), the liposomes contained either (i) 25 mM ANTS and 45 mM NaCl, (ii) 90 mM DPX, or (iii) 12.5 mM ANTS, 45 mM DPX, and 22.5 mM NaCl. For some experiments, the Tb/DPA assay was used (Wilschut et al., 1980). In these cases, the liposomes contained either (i) 2.5 mM $TbCl_3$, 50 mM NTA, and 20 mM NaCl, (ii) 50 mM DPA, or (iii) 1.25 mM $TbCl_3$, 25 mM NTA, 10 mM NaCl, and 25 mM DPA. All solutions were buffered with 10 mM glycine at pH 9.5. In all cases, the encapsulated solutions were isoosmotic to the buffers used for the column chromatography and in the leakage and fusion experiments. To measure the change in NBD fluorescence quantum efficiency, liposomes composed of DOPE-Me or DOPE/DOPC containing 0.1 mol % of NBD-PE were made in the isoosmotic NaCl/glycine buffer. The liposomes were extruded through polycarbonate membranes (Nuclepore Corp., Pleasanton, CA) with 0.1-μm pores and separated from unencapsulated material on Sephadex G-75 (Pharmacia) using 100 mM NaCl, 10 mM glycine, and 0.1 mM EDTA (pH 9.5) for the elution buffer. There was no significant binding of ANTS to the exterior of the liposomes (Ellens et al., 1984, 1985, 1986a).

The size distribution of the liposomes was measured using dynamic light scattering (Coulter Model N4; Coulter Electronics, Inc., Hialeah, FL). For the DOPE-Me liposomes used in the fusion studies, which were extruded once, the *Z*-average diameter was 270 ± 95 nm for the ANTS liposomes, 220 ± 75 nm for the ANTS/DPX liposomes, and 280 ± 100 nm for the DPX liposomes. The encapsulated volume was 3.2 μL/μmol of lipid for the ANTS/DPX-containing liposomes and 5 μL/μmol of lipid for the ANTS-containing liposomes. Since the area per DOPE-Me headgroup in the bilayer is about 60 Å^2 (Mulukutla & Shipley, 1984; Gruner et al., 1988), we know that the liposomes have two or more lamella. For the DOPE/DOPC (3:1) liposomes, which are extruded at least 4 times, smaller diameters were found, 170 ± 45 nm, and the encapsulated volumes were 2.3 ± 0.1 μL/μmol, for all of the encapsulated fluorophores of both assays. The repeated extrusion appears to produce more uniform liposomes, but they still must be oligolamellar.

The absolute rates of leakage and fusion varied between liposome preparations, but the temperature dependence of the kinetics was the same for all liposome preparations. The encapsulated contents of liposomes stored at pH 9.5 and 4 °C showed no measurable leakage for at least a week. All samples were used within 2 days.

Fusion Studies. Fluorescence and light scattering were measured with either a Spex Fluorolog 2 (Spex Industries, Edison, NJ) or an SLM 4000 fluorometer (SLM Instruments, Champaign—Urbana, IL) equipped with two 90° emission channels, allowing both fluorescence and light scattering to

[2] Throughout this work, the terms isotropic state and ΔT_I are defined operationally by the presence of the narrow isotropic ^{31}P NMR resonances and the temperature range over which these resonances are observed, respectively. For some lipid systems, these resonances are associated cubic-phase X-ray diffraction patterns (Luzzati & Reiss-Husson, 1966; Rilfors et al., 1986; Gruner et al., 1988; Siegel et al., 1989b).

be monitored simultaneously. With the ANTS/DPX fusion assay, mixing of aqueous contents of ANTS- and DPX-containing liposomes is registered as a decrease in ANTS fluorescence due to quenching of ANTS by DPX (Ellens et al., 1985). Leakage is measured with liposomes containing both ANTS and DPX (Ellens et al., 1984). The fluorescence scale was calibrated with the intensity of the mixture of ANTS and DPX liposomes in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5) taken as 100% fluorescence (0% fusion). The 0% fluorescence level was set to the intensity of the liposomes containing the coencapsulated ANTS/DPX, i.e., the leakage liposomes. Excitation was at 360 nm and emission >530 nm. On the figures, fusion (percent maximum Q) is equal to 100 minus the recorded fluorescence, which is equal to the percentage of ANTS which is quenched by DPX at that time. ANTS fluorescence is not sensitive to pH between 9.5 and 4.5, and the quenching efficiency of DPX is not affected by pH in this region.

The normal procedure for calibrating the 100% fluorescence intensity level for the Tb/DPA fusion assay (Wilschut et al., 1980; Düzgüneş & Bentz, 1988) could not be used at high temperatures. Briefly, this procedure calls for lysing (e.g., 50 μM lipid) of Tb-containing liposomes (type i above) in an EDTA-free buffer containing 20 μM free DPA, which is excess DPA at 20 °C. At room temperature, this fluorescence intensity is equal to that of 100 μM (lipid) Tb/DPA-containing liposomes (type iii above), since the encapsulation volumes are essentially identical. As the temperature is increased, the absolute intensity of these two standards decreased, and most importantly, the intensity of the free Tb/DPA solution decreased far more rapidly than did that of the liposome-encapsulated Tb/DPA, especially above 50 °C. Part of this decrease may reflect a diminished quantum efficiency. However, it is very likely that the *difference* between the free solution and the liposome-encapsulated Tb/DPA is due to dilutional dissociation of the Tb/DPA complex in the free solution. Lysing the liposomes dilutes the Tb concentration about 4000 times. The simple mass action complexation may be expected to show increased dissociation as the temperature increases.

Since the nonleaky fusion of a Tb-containing and a DPA-containing liposome would encapsulate the same solution as is in the Tb/DPA-containing liposomes, we have chosen to calibrate the 100% levels using the Tb/DPA-containing liposomes, equilibrated to the appropriate temperature. The alternative approach of increasing the DPA concentration in the free solution with the lysed Tb-containing liposomes is untenable due to excess light adsorption by the DPA. Using the free solutions for calibration would certainly overestimate the extent of contents mixing since the "100%" would be set artificially low.

The fusion was started by injection of an aliquot of the various stock solutions into a magnetically stirred cuvette, containing 2.0 mL of the liposome suspension in glycine buffer (100 mM NaCl, 2 mM glycine, and 0.1 mM EDTA, pH 9.5). To achieve pH 7.4, the aliquot was 20–30 μL (depending upon the temperature) of a 1.0 M TES/pH 7.0 buffer. To achieve pH 7.4 with 5 mM Mg^{2+}, the aliquot was 25 μL of a 0.4 M $MgCl_2$/1.0 M TES/pH 7.0 buffer. To achieve pH 9.5 with 20 mM Mg^{2+}, the aliquot was 25 μL of a 1.60 M $MgCl_2$/pH 6.8 buffer. To achieve pH 4.5, the aliquot was 20 μL of a 5 M acetic acid/acetate/pH 4.5 buffer. All final pH values were measured.

Differential Scanning Calorimetry. To ensure full and uniform hydration, samples for DSC, NMR, and X-ray diffraction studies were all made according to the following protocol. In two-component systems, either lipids were weighed out as powders and cosolubilized in chloroform or the chloroform solutions of the component lipids were mixed. Lipid concentrations in multicomponent systems are accurate to ±2%. Chloroform was removed via rotary evaporation for 30 min at room temperature under high vacuum. The lipid was hydrated (<6 mg/mL) for 2–3 h at 4 °C, extensively vortexed, and then subjected to three freeze–thaw cycles (dry ice and a water bath at 50 °C, respectively). The lipid was then pelleted for 20 min in an ultracentrifuge (25 000 rpm in an SW-40 Ti rotor; 80000g) at 4 °C and resuspended at the final concentration. If the buffer employed contained divalent cations, the sample was pelleted and resuspended in a new 10-mL aliquot of buffer after each freeze–thaw cycle. This procedure yielded the same DSC results as samples dialyzed in excess buffer for over 24 h. If the sample made up in this manner was frozen for later use (at −70 °C, as occasionally occurred prior to NMR or X-ray experiments), the samples were subjected to three freeze–thaw cycles just prior to examination. Samples not treated in this manner occasionally exhibited isotropic ^{31}P NMR resonances at lower temperatures than samples prepared immediately before use. This type of hysteresis has been noted previously (Gruner et al., 1988).

The buffers were all 100 mM in NaCl, containing in addition either (i) 50 mM acetate/0.1 mM EDTA at pH 4.5; (ii) 20 mM $MgCl_2$, 10 mM glycine at pH 9.5 or 9.9, and 0.1 mM EDTA; or (iii) 5 mM $MgCl_2$, 10 mM TES at pH 7.4, and 0.1 mM EDTA. Thermograms were obtained by using a Microcal MC-2 calorimeter at a scan rate of either 13 or 27 °C/h. There was no significant difference in the onset or peak temperatures of the L_α/H_{II} phase transition between thermograms obtained at these two scan rates. For DOPE-Me at neutral or low pH, we found a small low-temperature shoulder to the transition in most, but not all, of the thermograms when the scan rate was 13 °C/h. The significance of this shoulder is discussed below.

X-ray Diffraction. Samples were prepared and equilibrated as above. X-ray diffraction patterns were obtained at station 7.3 of the synchrotron radiation source at the SERC Daresbury Laboratory, using 0.150-nm radiation and a linear detector constructed at the Daresbury Laboratory, as described previously (Nave et al., 1985; Tenchov et al., 1987). The samples were placed between mica windows in a brass chamber thermostated via an external circulating water bath. The temperature of the sample cell was continuously monitored via a thermocouple. The temperature of the sample cell was increased in an approximately linear fashion at ca. 9 °C/min as a total of 255 consecutive diffraction patterns were obtained, each pattern being compiled in 1.2 s with a dead time of 50 μs between frames. The acquired data were stored in a VAX-11/750 computer and corrected for detector response by comparison with a pattern recorded using a fixed source and averaged over several hours. Data were smoothed with the OTOKO program, which uses a cubic spline interpolation subroutine. Static X-ray patterns were obtained at constant temperature using a Rigaku RU-100H rotating-anode X-ray source and a flat-film camera.

^{31}P NMR. Samples were prepared and equilibrated as above. ^{31}P nuclear magnetic resonance (NMR) spectra were obtained as described previously (Yeagle & Sen, 1986) with a JEOL FX270 Fourier-transform spectrometer with a broad-band probe in 10-mm tubes. A total of 2000 scans were collected with a repetition rate of 1 s. A fully phased cycled (32 pulse) Hahn echo sequence was used with a 40-μs echo.

The echo sequence eliminates base-line artifacts, removing the need for first-order phase corrections (Rance & Bird, 1983). Data were collected prior to the refocusing of the echo and the FID transformed from the top of the echo. This procedure avoids artifacts that arise when the receiver is turned on only at the expected refocusing of the echo. Because of the short echo time that must be employed to capture all the resonance intensity (due to the very short T_2°), the finite length of the transmitter pulses ($\pi/2$ = 10 μs) must be taken into account in determining the refocusing point of the echo. Because of the very rapid decay in the FID of the broad component in these spectra, care must be taken to transform from the top of the echo because the broad features are readily lost in the Fourier transform if the refocusing point of the echo is missed. The ^{1}H decoupler was gated on during acquisition and off the remainder of the time to prevent sample heating. The effectiveness of the decoupling was improved by using single-frequency decoupling, rather than noise-modulated decoupling, with the frequency set at the resonance frequency of the phospholipid headgroup protons. Exponential line broadening of 50–200 Hz was used. Recalculations showed no introduction of artifacts occurred in this range.

Freeze–Fracture Electron Microscopy. DOPE–Me (50 μmol) and NBD-PE (0.05 μmol) were mixed in chloroform and dried down in a rotary evaporator for 1 h under high vacuum. The lipid was hydrated in 10 mL of glycine buffer (10 mM glycine/100 mM NaCl/pH 9.5) by brief sonication and vortexing, followed by one freeze–thaw cycle. The micrographs of the MLV were of these liposomes. For the LUV, a portion of these liposomes were then extruded through a 0.4-μm filter and 4 times through a 0.2-μm filter at 120 psi (Hope et al., 1985).

To lower the pH, 75 μL of 2 M acetate/acetic acid stock (pH 4.5) was injected into 3 mL of a 200 μM liposome suspension at 55 °C. To achieve 20 mM Mg^{2+}/pH 9.5 (at 45 °C), 60 μL of 1.0 M $MgCl_2$/pH 6.8 stock was injected into the cuvette. The NBD fluorescence and 90° light scattering were followed until the LUV suspension showed "collapse" (~2 min), as described in Figure 4. The MLV suspension showed an increase in the intensity of scattered light, which was followed by a decrease in intensity, perhaps due to massive aggregation. Ten minutes after the time of LUV collapse, both LUV and MLV samples were cooled to room temperature for freeze–fracture.

Samples for freeze–fracture were taken directly from the cuvettes. A 0.1–0.3-μL aliquot, without cryoprotectant, was sandwiched between a pair of Balzers (Nashua, NH) copper support plates and rapidly plunged into liquid propane (Costello & Corliss, 1978). The specimen was fractured and replicated in a Balzers BAF 400 freeze–fracture unit at a vacuum of 3×10^{-7} mbar, or better, and at −115 °C. Replicas were floated off in HNO_3 (3.0 N) and washed in a graded series of Clorox solutions. Replicas were viewed on a Philips 300 electron microscope at magnifications of 6000–75000×.

Results

Structural Studies: DSC, X-ray Diffraction, and ^{31}P NMR. The onset and peak L_α/H_{II} transition temperatures for all the systems studied are given in Table I. The peak and onset temperatures were generally reproducible to within 2 °C or better, although DOPE/DOPC (3:1) in the presence of 20 mM Mg^{2+} at pH 9.5 yielded less reproducible temperatures for unknown reasons. Endotherms obtained at scan rates of 13 and 27 °C/h were the same within experimental error. For the DOPE-Me systems, the endotherms were relatively narrow, with full widths at half-height (FWHH) of 1.0–1.3 °C and transition enthalpies of ca. 200 cal/mol. For the DOPE/DOPC systems, the endotherms were more poorly defined, with FWHH of 3–5 °C and enthalpies of ca. 70 cal/mol for DOPE/DOPC (3:1) systems and FWHH at 4–8 °C and ca. 100 cal/mol for DOPE/DOPC (4:1) systems. The endotherms of the (4:1) systems were often skewed to the high-temperature side. Endotherms in the DOPE/DOPC (2:1) system were of very low amplitude, and the transition temperatures were poorly reproducible, ranging from 76 to 83 °C in peak temperature.

Table I: L_α–H_{II} Phase Transition Temperatures As Determined by DSC

lipid system	cations (all buffers contain 100 mM NaCl)	transition temp (°C) onset ± SD	transition temp (°C) peak ± SD
DOPE-Me	pH 4.5	64 ± 2	66 ± 2
	pH 7.4[a]	65 ± 2	66 ± 2
	pH 9.5/20 mM Mg^{2+}	58 ± 2	60 ± 2
	pH 7.4/5 mM Mg^{2+}	65 ± 2	66 ± 2
DOPE/DOPC (2:1)	pH 4.5	≥80[b]	
	pH 9.5/20 mM Mg^{2+}	≥80[b]	
DOPE/DOPC (3:1)	pH 4.5	56 ± 3	60 ± 4
	pH 9.5/20 mM Mg^{2+}	67 ± 4	71 ± 5
	pH 7.4/5 mM Mg^{2+}	59 ± 4	62 ± 4
DOPE/DOPC (4:1)	pH 4.5	43 ± 2	47 ± 2
	pH 9.5/20 mM Mg^{2+}	54 ± 2	56 ± 2

[a] The transition temperatures are the same if the buffer contains 150 mM NaCl. [b] Ill-defined small endotherm with an onset temperature of ≥80 °C.

Peak temperatures from the same lot of lipid were reproducible to within 1.0 °C. Different lots occasionally yielded transition temperatures which were as much as 3 °C different, although 2D TLC indicated no difference in purity (to 0.5 mol % accuracy). Most of the variability between the PE/PC mixtures can be ascribed to small variations in the PE mole fraction, where a 1% change can change T_H by 2–3 °C. The data cited here are averaged over several lot numbers.

The widths of the DOPE/DOPC system endotherms indicate that the L_α and H_{II} phases coexist over substantial temperature ranges. For the sake of simplicity, we will use the peak temperature of an endotherm as the value of T_H. However, the onset temperatures in Table I (obtained by extrapolation of the low-temperature side of the endotherm to the extrapolated base line) are more representative of the temperatures at which the H_{II} phase can begin to form in given systems.

T_H increases as the DOPE/DOPC mixtures contain more DOPC, as shown by Tilcock et al. (1982). For these mixtures, the dependence of T_H on PC mole fraction is roughly linear. For pure DOPE, $T_H \simeq$ 6–10 °C (Epand, 1985; Gagné et al., 1985).

Clearly, either cation (H^+ or Mg^{2+}) can neutralize the membrane surfaces sufficiently to permit the transition. H^+ binds to the amino group, yielding a zwitterionic headgroup [$pK_a \sim 9.5$ for DPPE in DPPC SUV; (Tsui et al., 1986)]. Mg^{2+} presumably binds to the phosphate (McLaughlin et al., 1981; Lau et al., 1981). It is interesting that for DOPE-Me, the T_H is lower with Mg^{2+} than with H^+, whereas the opposite result is found with the DOPE/DOPC mixtures. This effect should be useful for elucidating the role and position of cation binding on the thermodynamics of the phase transition. On the other hand, the T_H is essentially the same for DOPE-Me at pH 4.5 and at pH 7.4 with 5 mM Mg^{2+} in the medium (Table I). Previously, we found that pH 4.5 and 7.4 gave essentially identical T_H's (Ellens et al., 1986b); thus, the presence of 5 mM Mg^{2+} at pH 7.4 has little effect upon T_H.

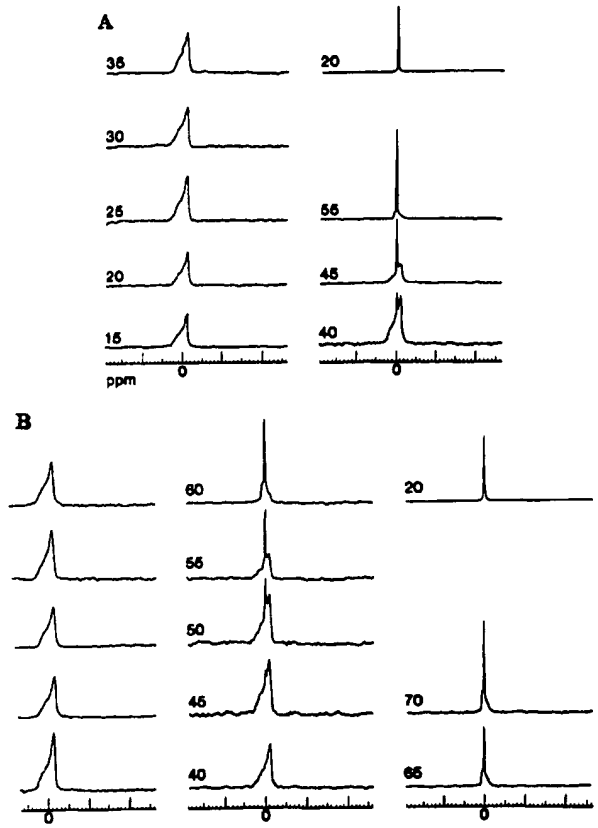

FIGURE 1: ^{31}P NMR spectra are shown for DOPE-Me at pH 9.5 in the presence of 20 mM Mg^{2+} (A) and for DOPE/DOPC (3:1) at pH 7.4 in the presence of 5 mM Mg^{2+} (B). The spectra were obtained by scanning from lower to higher temperatures, except the top right spectra which were obtained by cooling the same sample back down to 20 °C. Interestingly, in all cases, the signal after cooling back to 20 °C is completely isotropic. Major divisions of the indicated scale of chemical shifts represent 100 ppm.

We obtained time-resolved X-ray diffraction patterns for several of the systems studied calorimetrically. This was done to verify that the observed transitions were to the H_{II} phase.

The samples studied were DOPE-Me at pH 4.5 and 7.4 and with 20 mM Mg/pH 9.5 and DOPE/DOPC (3:1) at pH 4.5 and with 5 mM Mg/pH 7.4. The diffraction patterns indicate the existence of no lattices other than L_α and H_{II}. Of course, this does not rule out the formation of short-lived or randomly dispersed structures, neither of which would provide coherent reflections. There are several reports of inverted cubic phase X-ray diffraction patterns being observed in similar systems, but only after long time periods (Gruner et al., 1988; Shyamsunder et al., 1988; Siegel et al., 1989b). Thus, we would not expect these patterns to arise during the rapid scans (9 °C/min) used in this study. We noted that upon cooling certain samples below T_H, the diffraction patterns showed that the sample did not return to a well-ordered L_α phase, as had been found also by Gruner et al. (1988).

^{31}P NMR indicates the presence of additional structures. Figure 1 shows spectra for DOPE-Me with 20 mM Mg/pH 9.5 (A) and DOPE/DOPC (3:1) with 5 mM Mg/pH 7.4 (B). At the lower temperatures in each sample, the NMR spectra show the axially symmetric powder pattern characteristic of phospholipids in L_α phases (Cullis & Hope, 1978). The most striking feature of these spectra is the emergence of an isotropic resonance at 35 °C for DOPE-Me and at 45 °C for DOPE/DOPC (3:1), 25 and 12 °C below the T_H of the systems, respectively. It is possible that these resonances mark the first step in the formation of an inverted cubic phase, such as that reported in DOPE-Me at neutral pH (Gruner et al., 1988; Siegel et al., 1989b). Formation of this phase is often not evident in DSC data because of the long incubation times necessary for the appearance of macroscopic amounts of it and because of the trivial enthalpies ($\ll kT$) found in samples at the lipid concentrations used (Siegel, 1986c; Gruner et al., 1988; Siegel et al., 1989b). In both of the systems in Figure 1, a ^{31}P NMR powder pattern characteristic of the H_{II} phase emerges (Cullis & Hope, 1978) at temperatures close to the L_α/H_{II} transition temperature determined via DSC, i.e., at ca. 55 °C in (A) and 60 °C in (B), as compared to DSC transition peak temperatures of 60 and 62 °C, respectively (Table I).

Table II: Onset Temperatures of Isotropic ^{31}P NMR Resonances[a]

lipid	cations[b]	T_I (°C)
DOPE-Me	pH 4.5	40–45[c]
	pH 7.4	30–35,[d] 40–50[c]
	5 mM Mg/pH 7.4	45–50[c]
	20 mM Mg/pH 9.5	35–40[c]
DOPE/DOPC (2:1)	pH 7.4	65–70[f]
DOPE/DOPC (3:1)	5 mM Mg/pH 7.4	45–50[c]
DOPE/DOPC (4:1)	pH 7.0	<35[e]

[a] These temperatures are not phase transition temperatures and can be very sensitive to equilibration techniques and contamination. Data produced in this study did give reproducible ΔT_I values.[3] [b] The media also contain 0.1–0.2 M NaCl unless otherwise noted, and the sample is in excess water. [c] This work. [d] Gagné et al. (1985). [e] Tilcock et al. (1982). [f] van Duijn et al. (1986).

When the hexagonal phase samples were cooled back to 20 °C, the result is a pure isotropic resonance; i.e., the samples do not return to a well-ordered L_α phase. It has been noted previously that repeated freeze–thaw cycles are required to reestablish long-range order in the L_α phase (Cullis et al., 1978b; Ellens et al., 1986b; Gruner et al., 1988).

The ^{31}P NMR resonance is extremely sensitive with respect to the amount of lipid which is involved in isotropic motion. By measuring the area under the curves, we can make a rough estimate of these percentages. Because of the narrowness of the ^{31}P NMR resonances, it is possible to detect such components when they arise from as little as a few percent of the total phospholipid. In Table II, we have collected the onset temperatures (T_I) for isotropic ^{31}P NMR resonances for the lipid systems. For example, Figure 1A gives T_I = 35–40 °C for DOPE-Me with 20 mM Mg/pH 9.5.[3]

Fusion Studies. Liposomes were made by the REV procedure and extruded through 0.1-μm Nucleopore filters (Ellens et al., 1986a,b). Fusion was monitored by the Tb/DPA or the ANTS/DPX assay for the mixing of encapsulated contents within the fused liposomes. Figure 2A shows the fusion of DOPE/DOPC (3:1) liposomes induced with 5 mM Mg^{2+} at pH 7.4, using the Tb/DPA assay. Figure 2B shows the decrease of fluorescence intensity due to the dissociation of preencapsulated Tb/DPA complex. This decrease is due to both the leakage of contents and the influx of medium into the fusing liposomes (Bentz et al., 1985a; Düzgüneş & Bentz, 1988). In both instances, the Tb/DPA complex is disrupted by EDTA and Mg^{2+} in the medium. In this case, there is little

[3] Tilcock et al. (1982) found that the isotropic resonance intensity increased markedly in the first 30 min, following a 10-min equilibration. However, Boni and Hui (1983) did not observe any changes in the intensity of the isotropic resonance over the course of 6 h. Likewise, for the data presented here, there was no evidence of time evolution. In several cases, a second ^{31}P NMR scan was taken at the same temperature (each one requiring about 15 min), and we could find no significant difference between them. It is clear that a rigorous equilibration routine is crucial for these types of experiments. Gruner et al. (1988) reached the same conclusion.

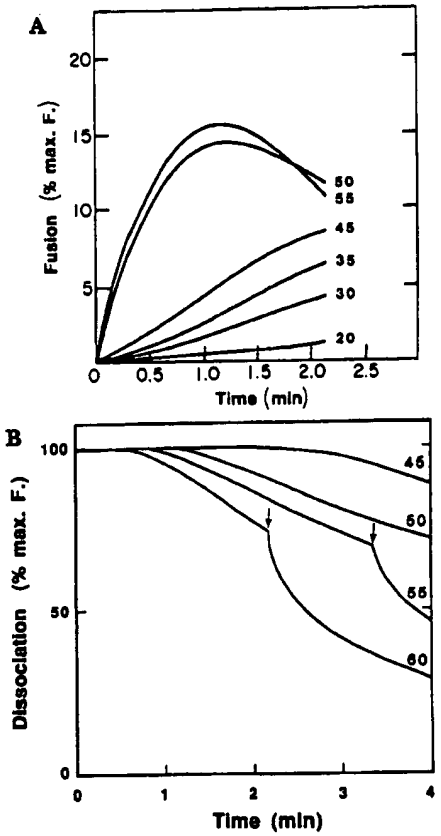

FIGURE 2: Temperature dependence of the fusion and leakage of DOPE/DOPC (3:1) liposomes at pH 7.4 with 5 mM Mg^{2+}. Panel A shows the fusion measured using the Tb/DPA assay for mixing of aqueous contents. The temperature is shown beside each curve in degrees centigrade. Panel B shows the loss of fluorescence intensity of preencapsulated Tb/DPA complex due to the leakage of contents and the influx of medium following the fusion of these liposomes. The Tb/DPA complex is dissociated by the competitive chelation of Tb^{3+} by EDTA and of DPA by Mg^{2+}. The arrows in panel B mark the collapse event, a sharp ejection of liposomal contents, which is described in detail in Figures 4–6, where the arrows also mark the collapse in those figures.

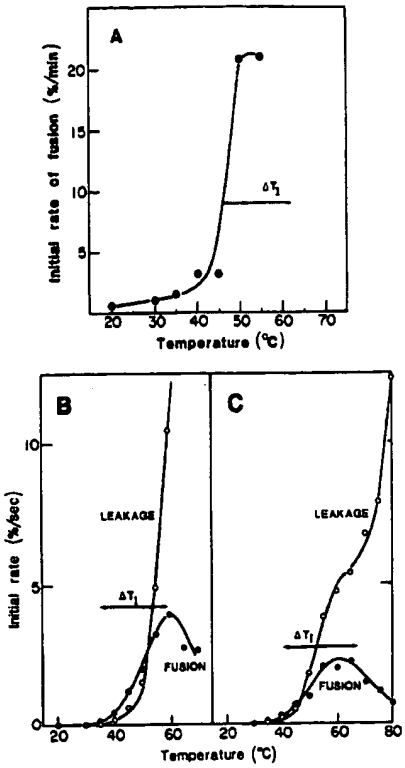

FIGURE 3: Initial rates of fusion of DOPE/DOPC (3:1) liposomes in 5 mM Mg^{2+}/pH 7.4 (A) and of DOPE-Me liposomes in 20 mM Mg/pH 9.5 (B) and at pH 4.5 (C). The initial rate is the initial slope of the fusion curve, as in Figure 2A. The temperature range of the isotropic ^{31}P NMR resonances is demarked by ΔT_I, which ends at the DSC-measured hexagonal phase transition temperature, T_H. Fusion is promoted in the temperature range of the isotropic ^{31}P NMR signal. Fusion for the DOPE-Me liposomes was measured by using the ANTS/DPX contents mixing assay.

leakage initially. However, over time, the leakage increases, especially above 45 °C which is the beginning of ΔT_I. The ANTS/DPX assay system gave the same qualitative results, except that leakage is more rapid initially. We believe this difference is due to the liposome preparations, rather than some discrepancy between the assays. The absolute rates of fusion and leakage varied between liposome preparations, even when the same assay system was used. For DOPE-Me liposomes containing 2 mol % diacylglycerol in pH 7.4/5 mM Mg^{2+}, both assays showed qualitatively the same behavior [see Siegel et al., 1989a)]. A detailed comparison of the two assays when encapsulated in the same liposome is currently in progress (Alford et al., 1989).

Often at the higher temperatures, there is an eventual abrupt loss of fluorescence due to an ejection of contents following "collapse" of the fusing structures. We will describe this event in fuller detail in the next section.

In Figure 3, the initial rate of fusion (i.e., the initial slope of the fluorescence curves) is plotted versus temperature for three systems. The temperature ranges where isotropic ^{31}P NMR resonances are found, ΔT_I, are shown (Table II).

For DOPE/DOPC (2:1), similar results have been found previously. At pH 4.5, there is a sharp increase in the fusion rate at ~60 °C and a decrease in the rate at ~80 °C (Ellens et al., 1986b). Subsequently, van Duijn et al. (1986) found at pH 7.4 that ΔT_I begins at ~60 °C, using ^{31}P NMR. Their NMR data also showed no H_{II} phase resonances up to 70 °C. ΔT_I for DOPE-Me is insensitive to pH in the range of 4.5–7.4 (Table II). To the extent that this is also the case for DOPE/DOPC (2:1), these data confirm the speculation in Ellens et al. (1986b) that the increase in fusion kinetics at 60 °C was correlated with the onset of the isotropic resonances. These liposomes also showed severalfold slower fusion from 40 to 60 °C, where the equilibrium phase is L_α. As discussed below, we believe that this fusion is due to another mechanism.

For all of these systems, it is obvious that when the temperature reaches ΔT_I, there is a sharp increase in the rate of fusion and in the rate of leakage. When the temperature reaches T_H, the liposomes undergo contact-mediated lysis, and fusion is abolished (Ellens et al., 1986a,b; Bentz et al., 1987).

Collapse following Fusion. In Ellens et al. (1986b), we described a unique phenomenon associated with the fusion of DOPE-Me liposomes. After a certain time period of fusion, there is an abrupt ejection of the liposomal contents into the medium, which we refer to as collapse. Figure 2B shows this abrupt leakage with DOPE/DOPC (3:1) liposomes fusing in 5 mM Mg^{2+} at pH 7.4, as monitored by the dissociation of preencapsulated Tb/DPA complex. While we define the event by the abrupt ejection of aqueous contents, we have found that the scattered light intensity abruptly changes at the same time. In those instances where collapse occurs after significant gradual leakage, the abrupt change in the light scattering is the clearest indication that collapse has occurred.

The collapse phenomenon can also abruptly change the quantum yield of fluorescently labeled lipids. Figure 4 shows

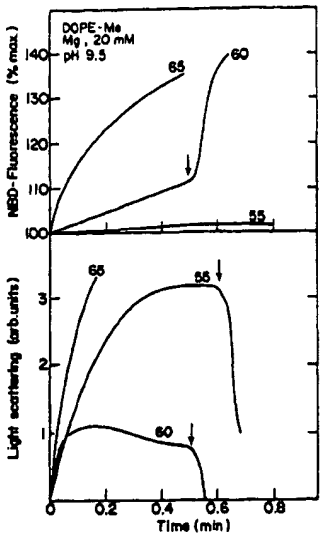

FIGURE 4: Time course of NBD fluorescence increase in DOPE-Me liposomes with 20 mM Mg/pH 9.5 at various temperatures. The upper panel shows the fluorescence curves, and the lower panel shows the corresponding 90° light-scattering traces. It is clear that the collapse, at higher temperatures, can signal a rapid increase in NBD fluorescence. The fluorescence scale is set with buffer at 0% and the initial fluorescence (pH 9.5 without Mg^{2+}) at 100%. The arrows mark the onset of the collapse event.

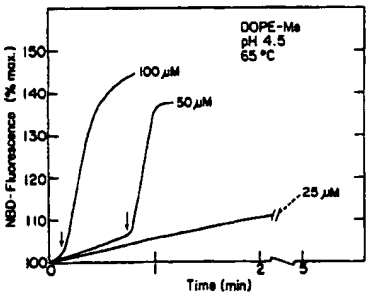

FIGURE 5: Effect of lipid concentration on the collapse time as monitored by NBD fluorescence. The arrows mark the onset of the collapse event.

this event using DOPE-Me liposomes with 20 mM Mg/pH 9.5. Here we monitored the 90° light scattering (lower panel) and the fluorescence of NBD-PE incorporated into the liposomes at 0.1 mol %, i.e., where there is no energy transfer or self-quenching (Struck et al., 1981). Under these conditions, the NBD quantum efficiency can be used to monitor the transition to the H_{II} phase (Ellens et al., 1986a,b; Bentz et al., 1987; Hong et al., 1988). At 65 °C ($>T_H$), the NBD quantum efficiency immediately increases (upper panel), as the liposomes begin transforming to H_{II} phase precursors upon contact (Ellens et al., 1986a,b). At 60 °C ($\sim T_H$), there is a slow increase in fluorescence until ~0.5 min when the collapse demarks a rapid transition to inverted phase aggregates. There is a simultaneous abrupt decrease in the 90° light-scattering intensity. There is also a loss of aqueous contents (data not shown). At 55 °C ($<T_H$), we see no significant change in NBD fluorescence after the 90° light scattering demarks the collapse.

We have found that collapse occurs after a considerable extent of aggregation/fusion has occurred. However, Figure 5 indicates that collapse may not be simply a matter of the aggregation/fusion products reaching a particular size. NBD fluorescence from DOPE-Me liposomes shows that collapse occurs faster with 100 μM lipid than with 50 μM lipid, as an aggregation-dependent event would show. However, with 25

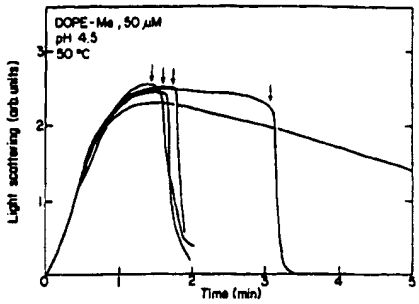

FIGURE 6: Ninety degree light scattering monitors the collapse of 50 μM DOPE-Me liposomes at pH 4.5 and 50 °C. The same experiment is repeated 5 times. There is a great deal of variability in the time at which collapse occurs. The arrows mark the onset of the collapse event.

μM lipid, the collapse did not occur even after 5 min. Simple kinetic explanations, such as a delay in the time of collapse due to the effect of aggregation reversibility at low lipid concentrations (Bentz & Nir, 1981; Ellens et al., 1984), are not very compelling under these circumstances (Bentz et al., 1988).

Collapse is a rather hysteretic event. Figure 6 shows the 90° light scattering for DOPE-Me liposomes at pH 4.5 and 50 °C. Here we repeated the experiment 5 times under identical conditions. In three cases, collapse occurred at about the same time (1.5–2 min). In one case, the collapse took about twice as long (~3 min). Finally, in one case, there was no collapse after 5 min. The fact that the light-scattering intensities were initially identical is clear evidence that the hysteresis is not due to errors in sample mixing.

Electron Microscopy. We have examined the products of collapse using freeze-fracture electron microscopy on DOPE-Me liposomes. Figure 7 shows these results. The upper panels show products of LUV (~0.15-μm diameter, initially) taken 10 min after collapse due to incubation at pH 4.5 and 55 °C (panel a) and with 20 μM Mg/pH 9.5 and 45 °C (panel b). For comparison, we show the corresponding products when the initial liposome samples are handshaken MLV (~0.3–1.0-μm diameter, initially). The light scattering for the MLV increases and then decreases in intensity, due to extensive aggregation, but there is no evidence of the abrupt change we define as collapse. Panel c shows the MLV at pH 4.5, and panel d shows the effect of 20 mM Mg/pH 9.5. The bar indicates 150 nm. The MLV do show evidence of some lipidic particle formation, although there are large areas of lamellar structure. The lipidic particles are far more numerous in the LUV samples. Most importantly, there are large domains of well-ordered lipidic particles, which have been correlated with cubic phase formation (Hui et al., 1983; Boni & Hui, 1983; Rilfors et al., 1986). We also note that the dimpled surfaces appear to show a few bilayers covering the domains of aligned lipidic particles.

The differences in the morphology between the LUV and MLV samples are due primarily to differences in size and/or the number of lamella per liposome. Gruner et al. (1988) found that adding a few mole percent of lyso-PE and fatty acid, to simulate phospholipid degradation products, catalyzed the cubic phase formation for DOPE-Me. This is not an issue for our experiments, since the MLV and the LUV were treated identically. Significant lipid degradation is unlikely in our experiments, where the lipids are exposed to the elevated temperatures for less than 15 min. In the pH 9.5/20 mM Mg^{2+} buffer, it might be argued that the Mg^{2+} has less access to the interior of the MLV, relative to the LUV. However, in this case, the difference between the MLV and the LUV

samples is less than that observed at pH 4.5. Furthermore, at pH 4.5, all of the lipid in both the MLV and the LUV was rapidly exposed to protonation due to the rapid permeation of the acetic acid component of the buffer [ca. 2 s (Barbet et al., 1984; Bentz et al., 1987)].

Discussion

For several inverted phase forming lipids which show isotropic ^{31}P NMR resonances, the same set of intermembrane intermediates appear to produce both liposome fusion and a lipid phase transition.[2] We have shown this by correlating the temperature dependence of liposome fusion kinetics with that of the appearance of ^{31}P NMR isotropic resonances in the bulk dispersion of the lipid.

We believe that a plausible rationale can be constructed from the current knowledge of the phase behavior of these lipids and recent theoretical calculations about the dynamics of the transition from the L_α phase to the H_{II} phase. First, we will discuss the phase behavior of these inverted phase forming lipids and how our model of the phase transition explicates this behavior. Next, we will show how the fusion data and the theoretical model of the phase transition dynamics can be combined in a unified description.

Phase Behavior of the Lipid Systems. DSC experiments on the systems listed in Table I show a low-enthalpy transition consistent with an L_α/H_{II} phase transition in each system. The transition temperatures found for DOPE-Me at pH 4.5 or 7.4 agree well with the values reported by Gagné et al. (1985), Ellens et al. (1986b), and Gruner et al. (1988), and the value for DOPE/DOPC (3:1) at pH 4.5 (60 °C) agrees well with that of Tate and Gruner (1987) of 50–55 °C for a mixture slightly richer in PE (DOPE/DOPC = 10:3 mol/mol).

The X-ray diffraction patterns confirm that the calorimetrically observed transitions were to the H_{II} phase. The identity of the phases in (3:1) DOPE/DOPC is as found in (10:3) DOPE/DOPC (Tate & Gruner, 1987), and the lattice constants of the two phases agree well with the values in that report (59 Å in the L_α phase at 30 °C in both cases, 73 vs 71 Å in the H_{II} phase at 80 °C). There is no rigorous evidence in these diffraction patterns for the existence of other phases in these systems across the entire temperature range. However, we did find a slight broadening of the L_α phase diffraction peaks upon heating (most evident in the second-order peaks) which is consistent with the beginning of a transition to an inverted cubic phase (Gruner et al., 1988).

The appearance of narrow isotropic resonances in the ^{31}P NMR spectra of multilamellar lipid preparations is a very sensitive indicator for the presence of isotropic or inverted cubic phases.[4] Our own measurements and those of others indicate the presence of isotropic resonances in all these systems over temperature ranges beginning from 10–30 °C below T_H (see Table II).

Correlation of the Fusion Mechanism with the Phase Transition. As shown in Figure 3, the initial rates of fusion and contents leakage in the DOPE/DOPC (3:1) system at neutral pH and in the DOPE-Me system at pH 4.5 or in the presence of 20 mM Mg^{2+}/pH 9.5 all respond in the same way as the temperature is raised through ΔT_I and T_H. The initial rates of fusion all increase dramatically as the temperature passes through the beginning of ΔT_I (Table II), plateaus or goes through maxima at temperatures either just below or at T_H, and decreases thereafter. Initial leakage rates also start to increase within ΔT_I but increase most dramatically (and surpass the rates of fusion) at temperatures $\geq T_H$.

These data agree with our previous results in that fusion sharply increases as the temperature reaches ΔT_I, for those systems which show isotropic resonances, and lysis sharply increases as the temperature reaches T_H (Bentz et al., 1985b; Ellens et al., 1986a,b). The observation that lipid mixing between the liposomes is rapid and extensive at temperatures above T_H is easily understood, as the membranes are being transformed to H_{II} phase structures. We do not call this process fusion, since there is no contents mixing involved.

Model of the $L_\alpha/I_{II}/H_{II}$ Phase Transition. Our data are consistent with a kinetic model of the L_α/H_{II} phase transition mechanism which is shown in Figure 8 (Siegel, 1986a–c; Siegel et al., 1988). Inverted micellar intermediates (IMI) formed between aggregated liposomes make the interfaces of the two membranes continuous, leading to lipid exchange in the outer monolayers. IMI formation is analogous to a subcritical fluctuation and should begin at temperatures well below T_H, particularly in systems with small L_α/H_{II} transition enthalpies like those described here. At T_H, IMI are proposed to aggregate in the plane of the two apposed membranes, forming other structures that result in rapid H_{II} phase formation and leakage (Siegel, 1986b).

In a particular subset of systems, where the ratio of the areas per lipid headgroup in the L_α and H_{II} phases is less than about 1.2 (Siegel, 1986b), or, equivalently, with "intermediate" values of the spontaneous radius of curvature described by Gruner et al. (1988), the IMI can form large numbers of another type of intermediate, denoted as an interlamellar attachment (ILA). ILA should yield isotropic ^{31}P NMR resonances, morphology like that of "lipidic particles" in freeze–fracture electron micrographs, and can assemble into inverted cubic phases (Siegel, 1986c). ILA formation should result in membrane fusion with mixing of aqueous contents. Hence, one expects to observe at least some fusion in unilamellar liposome dispersions under the same conditions in which bulk lipid–water samples exhibit isotropic ^{31}P NMR resonances. This is the case.

This model formally explains why only a subset of inverted phase forming lipids can form large numbers of form ILA, which we argue are responsible for the isotropic NMR resonances. It also predicts the formation of *some* ILA for *any* inverted phase forming lipid. Isotropic resonances are not always found for lipids which can achieve the H_{II} phase (Tilcock et al., 1984). However, Shyamsunder et al. (1988) have recently reported that pure DOPE, a lipid previously found to undergo a simple transition between the L_α and the H_{II} phase (Epand, 1985; Gagné et al., 1985), can be induced to form an inverted cubic phase by cycling the temperature above and below T_H several hundred times. Our model predicts that it is difficult for the ILA to revert to IMI (Siegel, 1986a), although the IMI can easily revert to apposed bilayers.

[4] The presence of <0.1-μm-diameter liposomes in the samples could also yield isotropic resonances. This is unlikely for two reasons. First, they would be fusing and would quickly reach sizes too large to produce isotropic motional averaging on the ^{31}P NMR time scale. Second, the samples were swelled from thin films of lipid, dispersed only by mild vortex mixing, and then pelleted. This should not produce any substantial number of small liposomes. We think that it is more likely that the isotropic resonances are produced by diffusion of the DOPE-Me molecules over the curved surfaces found in inverted cubic phases [e.g., see Siegel (1986c)]. Larsen et al. (1987) have shown that the motions of phospholipids with reorientational time constants of less than 20 μs produce isotropic ^{31}P NMR resonances in 109-MHz instruments. It can be shown that DOPE-Me diffusion on the surfaces of ILA (Figure 8) results in reorientational time constants of 4 μs or less, using a lipid diffusion coefficient for DOPE at 56 °C of 6×10^{-8} cm²/s (Rilfors et al., 1986).

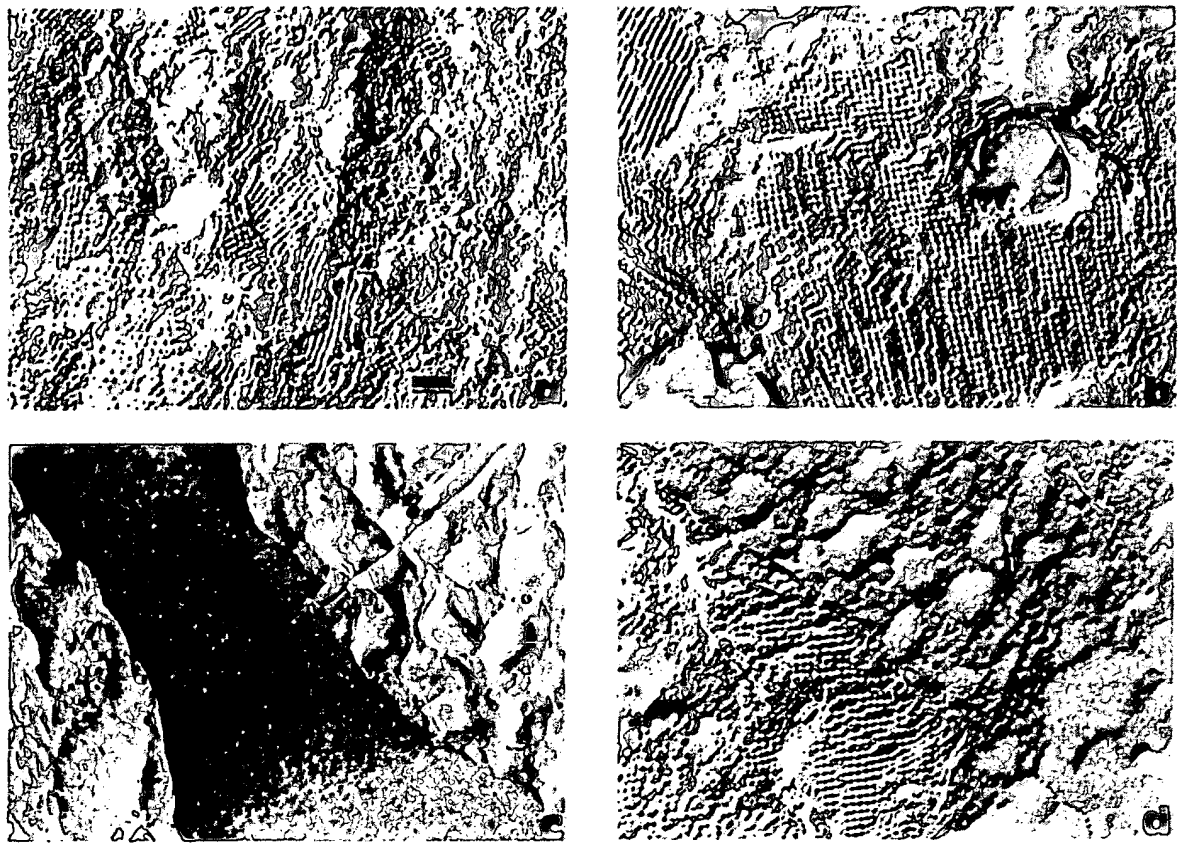

FIGURE 7: Freeze-fracture electron micrographs of DOPE-Me. Upper panels show the products of LUV (made by high-pressure extrusion at pH 9.5) obtained about 10 min after collapse due to lowering the pH to 4.5 at 55 °C (panel a) or adding 20 mM Mg/pH 9.5 at 45 °C (panel b). The lower panels show the corresponding cases when large MLV (diameter ~0.3–1.0 μm) are used initially. The MLV samples did not show collapse, but they were rapidly frozen after the same incubation time as the LUV samples. Thus, the MLV products in pH 4.5 at 55 °C (panel c) and with 20 mM Mg/pH 9.5 at 45 °C (panel d) are shown. In all cases, the lipid concentration was 200 μM. Therefore, the difference in the morphologies observed between the MLV and the collapse products of the LUV is due to the initial diameter and the number of lamella of the liposomes. All micrographs are at the same magnification. Bar: 150 nm.

Therefore, we would expect that each cycle through T_H would cause the accumulation of more ILA and the gradual formation of a cubic lattice, as has been found.

From the results of Shyamsunder et al. (1988), it is likely that the isotropic state/inverted cubic phase (I_{II}) can be induced in many, if not all, inverted phase forming lipids by the technique of exhaustively cycling the temperature through T_H. However, the correlation between the fusion mechanism and the isotropic state resides *only* in that subset of inverted phase forming lipids which show isotropic ^{31}P NMR resonances on the first heating scan. This is the situation which most closely corresponds to the initial contact between two liposomal membranes. The fact that pure DOPE requires hundreds of cycles through T_H to form many ILA, or whatever intermediates are responsible for the cubic lattices, simply proves that the initial interaction between two liposomes at $T \sim T_H$ is not likely to provoke the formation of enough ILA to produce measurable fusion.

In fact, not all of the fusion observed in PE-containing systems can be attributed to an ILA-mediated mechanism. Ellens et al. (1986a) showed that liposomes composed of DOPE, EPE, or TPE fused at temperatures below that of their respective values of T_H and that this fusion was abolished when the temperature exceeded T_H; i.e., only lysis and lipid mixing occurred. None of these lipids show isotropic resonances on the first heating scan. Furthermore, fusion could be observed as much as 35 °C below T_H, where no ILA and few, if any, IMI could form (Siegel, 1986b). The fusion observed 20 °C below ΔT_1 for DOPE/DOPC (2:1) in Ellens et al. (1986a) cannot be ascribed to an ILA-mediated process. At ΔT_1, where the fusion rate for this system sharply increased, the ILA-mediated fusion mechanism became dominant. Thus, while we have elucidated one molecular mechanism for fusion here, we have shown also that it is not the only mechanism.

We reach the same conclusion with respect to leakage mechanisms. We have found that leakage also increases within ΔT_1, which implies a mechanism which depends upon the number of IMI or ILA. The model described by Figure 8 would predict leakage only via H_{II}-domain formation, which can only occur at temperatures at and above T_H. The leakage mechanism far below T_H is not known. It may be that the accumulation of a few defects (IMI or ILA) in the area of membrane apposition increases membrane permeability and/or susceptibility to rupture. It is known that leakage also can occur when adhesive forces promote excessive membrane tension and rupture, which has been demonstrated for very large (diameter >2 μm) liposomes (Kachar et al., 1986; Evans & Needham, 1986; Niles & Cohen, 1987). The leakage mechanism within ΔT_1 may involve these forces, in addition to the defects formed by the IMI and ILA.

It has been proposed that lipidic particles have the same structure as we assign to IMI or ILA [see references in Verkleij (1984) and Cullis et al., (1985)]. Recently, ILA have been imaged directly using time-resolved cryotransmission electron microscopy (Talmon et al., 1989). Using the same technique, as well as freeze-fracture electron microscopy, Siegel et al.

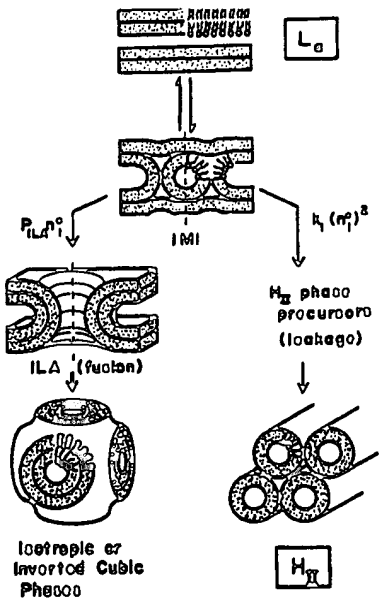

FIGURE 8: Proposed membrane–membrane interaction scheme. IMI are postulated *transient* intermediates in L_α/H_{II} phase transitions and can form at temperatures starting tens of degrees below T_H. H_{II} precursor formation is postulated to require aggregation of IMI between apposed bilayers at a rate $k_I n_I^{0\,2}$, where k_I is a rate constant and n_I^0 is the steady-state surface density of IMI. The rate of ILA formation from single IMI is $P_{ILA}n_I^0$, where P_{ILA} is a rate constant determined by lipid structural parameters. As detailed in Siegel (1986a,c), n_I^0 and k_I increase and P_{ILA} decreases with temperature, so that H_{II} phase precursor formation proceeds faster than ILA formation as T approaches T_H. P_{ILA} is a very sensitive function of the spontaneous radius of curvature of the lipid system (Siegel, 1986b,c; Gruner et al., 1988). To a first approximation, P_{ILA} tends to be large in systems with large H_{II} tubes.

(1989c) showed that the lipidic particles in DOPE-Me (pH 7.4) are ILA and that its inverted cubic phase probably forms from ILA.

Collapse and Inverted Cubic Phase Formation. The collapse phenomenon is defined by three consistent observations. First, it occurs after aggregation and fusion. Second, it only occurs at temperatures within or slightly above ΔT_I. The incubation time before collapse decreases as the temperature approaches T_H, and at or above T_H, aggregation leads immediately to lysis. Third, the event is very hysteretic, in that the collapse time is variable, especially at temperatures far below T_H and at low lipid concentrations (Figure 6).

Figure 7 shows that the collapse product of the LUV suspension contains extensive domains of ordered lipidic particles. Previous studies have shown that inverted cubic phases form in systems exhibiting ordered arrays of lipidic particles (Boni & Hui, 1983; Hui et al., 1983; Lindblom et al., 1986; Rilfors et al., 1986). Figure 7 also shows that MLV incubated at low pH produce far fewer lipidic particles and *no* ordered arrays. With 20 mM Mg^{2+}/pH 9.5, the MLV do show some domains of aligned lipidic particles, indicating that the transition is more rapid with bound Mg^{2+}. Together, these data imply that the inverted cubic phase can be most quickly assembled by the accretion of small units of bilayer.

The size of the lattice observed in Figure 7 is interesting. The square lattice formed by the lipidic particles has a center-to-center distance of ca. 29 nm. Siegel (1986c) predicted that ILA assemble inverted cubic phases via the formation of an intermediate lattice of roughly the size and geometry observed in these micrographs. The inverted cubic phases of DOPE-Me typically have different symmetries and smaller lattice constants (Gruner et al., 1988). Presumably, at temperatures closer to T_H, the ILA lattice transforms into these inverted cubic phases. With respect to this, we have observed a small additional endotherm at ca. 60 °C in most, but not all, thermograms of DOPE-Me obtained at neutral or low pH, when the scan rate was 13 °C/h. This endotherm is larger in samples with greater lipid concentrations, and at slower scan rates (Siegel et al., 1989a). On the other hand, inverted cubic phase X-ray diffraction patterns are observed only after 20-h incubations at temperatures above 60 °C and below T_H (Siegel et al., 1989b).

CONCLUSION

The model in Figure 8 predicts that fusion occurs in the temperature interval ΔT_I, where the principal fate of the IMI is ILA formation. This is the temperature dependence for fusion which we observe. Thus, for these systems, the molecular mechanism for fusion is well understood. Essentially, fusion is an obligatory step in the transition to the cubic phase for these systems.

It is worth recalling that even the well-studied Ca^{2+}-induced fusion of phosphatidylserine (PS) liposomes has resisted a rigorous structural characterization (Bentz & Ellens, 1988; Bentz et al., 1988). We believe the difficulty in the structural analysis of the PS/Ca^{2+} fusion mechanism is not that intermembrane intermediates (of a different type than those proposed here) do not exist but rather that in the cases thus far studied, the lifetime of the intermediate is too short to permit resolution.

Inverted phase forming lipids are found commonly in biological membranes [see references in Siegel (1987) and Bentz and Ellens (1988)]. Bacteria will alter the proportion of inverted phase forming lipids in their membrane in response to environmental stress (Lindblom et al., 1986; Goldfine et al., 1987). Physiological levels of lipid metabolites (e.g., diacylglycerols) drastically alter the stability of inverted phases and the kinetics of liposome fusion (Siegel et al., 1989a). The passive roles of lipids in biological membranes (e.g., as a permeability barrier) are reasonably well understood. What roles, if any, these inverted phase forming lipids play in the dynamic function of biological membranes (e.g., vesicle fusion during exocytosis) have yet to be determined.

ACKNOWLEDGMENTS

We thank Drs. John Silvius and Sol Gruner for stimulating discussions. D.P.S. is grateful to J. Banschbach for conscientious technical assistance, and D.P.S., L.J.L., and P.J.Q. are grateful to Dr. Colin Nave of the SERC Laboratory (Daresbury, U.K.) for expert aid in performing the time-resolved X-ray diffraction experiments. We also thank Andrea Mazel for the expert typing of the manuscript.

Registry No. DOPE, 2462-63-7; DOPC, 10015-85-7; DOPE-Me, 87803-74-5; Mg, 7439-95-4.

REFERENCES

Alford, D., Düzgüneş, N., & Bentz, J. (1989) *Biophys. J. 55*, 342a.

Barbet, J., Machy, P., Truneh, A., & Leserman, L. (1984) *Biochim. Biophys. Acta 772*, 347–356.

Bentz, J., & Nir, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1634–1637.

Bentz, J., & Ellens, H. (1988) *Colloids Surf. 30*, 65–112.

Bentz, J., Nir, S., & Wilschut, J. (1983) *Colloids Surf. 6*, 33–66.

Bentz, J., Düzgüneş, N., & Nir, S. (1985a) *Biochemistry 24*, 1064–1072.

Bentz, J., Ellens, H., Lai, M.-Z., & Szoka, F. C. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5742–5745.
Bentz, J., Ellens, H., & Szoka, F. C. (1987) *Biochemistry 26*, 2105–2116.
Bentz, J., Alford, D., Cohen, J., & Düzgüneş, N. (1988) *Biophys. J. 53*, 593–607.
Boni, L., & Hui, S. (1983) *Biochim. Biophys. Acta 731*, 177–185.
Borovjagin, V. L., Vergara, J. A., & McIntosh, T. J. (1982) *J. Membr. Biol. 69*, 199–212.
Caffrey, M. (1985) *Biochemistry 24*, 4826–4844.
Caffrey, M. (1987) *Biochemistry 26*, 6349–6363.
Campbell, R. F., Meirovitch, E., & Freed, J. H. (1979) *J. Phys. Chem. 83*, 525–533.
Costello, M. J., & Corliss, J. M. (1978) *J. Microsc. (Oxford) 112*, 17–37.
Cullis, P. R., & Hope, M. J. (1978) *Nature (London) 271*, 672–675.
Cullis, P. R., van Dijck, P. W. M., de Kruijff, B., & de Gier, J. (1978) *Biochim. Biophys. Acta 513*, 21–30.
Cullis, P. R., Hope, M. J., de Kruijff, B., Verkleij, A. J., & Tilcock, C. P. S. (1985) in *Phospholipids and Cellular Regulation* (Kuo, J. F., Ed.) pp 1–59, CRC Press, Boca Raton, FL.
de Kruijff, B., Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Mombers, C., Noordam, P. C., & de Gier, J. (1979) *Biochim. Biophys. Acta 555*, 200–209.
Düzgüneş, N., & Bentz, J. (1988) in *Spectroscopic Membrane Probes* (Loew, L., Ed.) Vol. 1, pp 117–159, CRC Press, Boca Raton, FL.
Düzgüneş, N., Paiemont, J., Freeman, K., Lopez, N., Wilschut, J., & Papahadjopoulos, D. (1984) *Biochemistry 23*, 3486–3494.
Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.
Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.
Ellens, H., Bentz, J., & Szoka, F. C. (1986a) *Biochemistry 25*, 285–294.
Ellens, H. Bentz, J., & Szoka, F. C. (1986b) *Biochemistry 25*, 4141–4147.
Epand, R. (1985) *Biochemistry 24*, 7092–7095.
Evans, E., & Needham, D. (1986) *Faraday Discuss. Chem. Soc. No. 81*, 267–280.
Gagnē, J., Stamatatos, L., Diacovo, T., Hui, S. W., Yeagle, P., & Silvius, J. (1985) *Biochemistry 24*, 4400–4408.
Goldfine, H., Johnston, N. C., Mattai, J., & Shipley, G. G. (1987) *Biochemistry 26*, 2814–2822.
Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665–3669.
Gruner, S. M. (1986) *Liposomes* (Ostro, M., Ed.) pp 1–38, Marcel Dekker, New York.
Gruner, S. M., Tate, M. W., Kirk, G. L., So, P. T. C., Turner, D. C., Keane, D. T., Tilcock, C. P. S., & Cullis, P. R. (1988) *Biochemistry 27*, 2853–2866.
Hong, K., Baldwin, P. A., Allen, T. M., & Papahadjopoulos, D. (1988) *Biochemistry 27*, 3947–3955.
Hui, S. W., Stewart, T. P., Boni, L. T., & Yeagle, P. L. (1981) *Science (Washington, D.C.) 212*, 921–922.
Hui, S. W., Stewart, T. P., & Boni, L. T. (1983) *Chem. Phys. Lipids 33*, 113–126.
Kachar, B., Fuller, N., & Rand, R. P. (1986) *Biophys. J. 50*, 779–788.
Kirk, G. L. (1984), Ph.D. Thesis, Princeton University, Princeton, NJ.
Kirk, G. L., Gruner, S. M., & Stein, D. L. (1984) *Biochemistry 23*, 1093–1102.
Larsen, D. W., Boylan, J. G., & Cole, B. R. (1987) *J. Phys. Chem. 91*, 5631–5634.
Lau, A., McLaughlin, A., & McLaughlin, S. (1981) *Biochim. Biophys. Acta 645*, 279–292.
Lentz, B. R., Carpenter, T. J., & Alford, D. R. (1987) *Biochemistry 26*, 5389–5397.
Leventis, R., Gagnē, J., Fuller, N., Rand, R. P., & Silvius, J. R. (1986) *Biochemistry 25*, 6978–6987.
Lindblom, G., Brentel, I., Sjölund, M., Wikander, G., & Wieslander, A. (1986) *Biochemistry 25*, 7502–7510.
Luzzati, V., & Reiss-Husson, F. (1968) *Nature (London) 210*, 1351–1352.
McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., & McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445–473.
Mulukutla, S., & Shipley, G. G. (1984) *Biochemistry 23*, 2514–2519.
Nave, C., Helliwell, J. R., Moore, P. R., Thompson, A. W., Worgan, J. S., Greenall, R. J., Miller, A., Burley, S. K., Bradshaw, J., Pigram, W. J., Fuller, W., Siddons, D. P., Deutsch, M., & Tregear, R. T. (1985) *J. Appl. Crystallogr. 18*, 396–403.
Niles, W., & Cohen, F. S. (1987) *Biophys. J. 90*, 703–735.
Nir, S., Bentz, J., & Wilschut, J. (1980) *Biochemistry 19*, 6030–6036.
Papahadjopoulos, D., Vail, W. J., Newton, C., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.
Rance, M., & Byrd, A. (1983) *J. Magn. Reson. 52*, 221–235.
Rilfors, L., Khan, A., Brentel, I., Wieslander, A., & Lindblom, G. (1982) *FEBS Lett. 149*, 293–298.
Rilfors, L., Eriksson, P.-O., Arvidson, G., & Lindblom, G. (1986) *Biochemistry 25*, 7702–7711.
Shyamsunder, E., Gruner, S. M., Tate, M. W., Turner, D. C., So, P. T. C., & Tilcock, C. P. S. (1988) *Biochemistry 27*, 2332–2336.
Siegel, D. (1986a) *Biophys. J. 49*, 1155–1170.
Siegel, D. (1986b) *Biophys. J. 49*, 1171–1183.
Siegel, D. (1986c) *Chem. Phys. Lipids 42*, 279–301.
Siegel, D. (1987) in *Cell Fusion* (Sowers, A. E., Ed.) pp 181–207, Plenum Press, New York.
Siegel, D., Ellens, H., & Bentz, J. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T., Hui, S. W., & Mayhew, E., Eds.) pp 53–71, Plenum Press, New York.
Siegel, D. P., Banschbach, J., Alford, D., Ellens, H., Lis, L. J., Quinn, P. J., Yeagle, P. L., & Bentz, J. (1989a) *Biochemistry* (following paper in this issue).
Siegel, D. P., Banschbach, J., Ellens, H., Alford, D., & Bentz, J. (1989b) *Biophys. J. 55*, 28a.
Siegel, D. P., Burns, J. L., Chestnut, M. H., & Talmon, Y. (1989c) *Biophys. J. 55*, 342a.
Silvius, J. R., & Gagnē, J. (1984a) *Biochemistry 23*, 3241–3247.
Silvius, J. R., & Gagnē, J. (1984b) *Biochemistry 23*, 3232–3240.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, B. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393–1401.
Szoka, F. C., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.
Talmon, Y., Burns, J. L., Chestnut, M. H., Siegel, D. P. (1989) *J. Electron Microsc. Tech.* (in press).

Tate, M. W., & Gruner, S. M. (1987) *Biochemistry 26*, 231-236.
Tenchov, B. G., Lis, L. J., & Quinn, P. J. (1987) *Biochim. Biophys. Acta 897*, 143-151.
Tilcock, C. P. S., Bally, M. B., Farren, S. B., & Cullis, P. R. (1982) *Biochemistry 21*, 4596-4601.
Tilcock, C. P. S., Hope, M. J., & Cullis, P. R. (1984) *Chem. Phys. Lipids 35*, 363-370.
Tsui, F. C., Ojcius, P. M., & Hubbell, W. L. (1986) *Biophys. J. 49*, 459-468.
van Duijn, G., Valtersson, C., Chojnacki, T., Verkleij, A., Dallner, G., & de Kruijff, B. (1986) *Biochim. Biophys. Acta 861*, 211-223.
Verkleij, A. J. (1984) *Biochim. Biophys. Acta 779*, 43-63.
Verkleij, A. J., van Echteld, C. J. A., Gerritsen, W. J., Cullis, P. R., & de Kruijff, B. (1980) *Biochim. Biophys. Acta 600*, 620-624.
Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.
Yeagle, P. L., & Sen, A. (1986) *Biochemistry 25*, 7518-7522.

Physiological Levels of Diacylglycerols in Phospholipid Membranes Induce Membrane Fusion and Stabilize Inverted Phases†

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Received April 29, 1988; Revised Manuscript Received August 31, 1988

ABSTRACT: In the preceding paper (Ellens et al., 1989), it was shown that liposome·fusion rates are substantially enhanced under the same conditions which induce isotropic ^{31}P NMR resonances in multilamellar dispersions of the same lipid. Both of these phenomena occur within the same temperature interval, ΔT_I, below the L_α/H_{II} phase transition temperature, T_H. T_H and ΔT_I can be extremely sensitive to the lipid composition. The present work shows that 2 mol % of diacylglycerols like those produced by the phosphatidylinositol cycle in vivo can lower T_H, ΔT_I, and the temperature for fast membrane fusion by 15-20 °C. N-Monomethylated dioleoylphosphatidylethanolamine is used as a model system. These results show that physiological levels of diacylglycerols can substantially increase the susceptibility of phospholipid membranes to fusion. This suggests that, in addition to their role in protein kinase C activation, diacylglycerols could play a more direct role in the fusion event during stimulus–exocytosis coupling in vivo.

Diacylglycerols are second messengers produced in cellular membranes by the PI cycle (Majerus et al., 1984, 1986; Sekar & Hokin, 1986) at levels of about 1 mol % with respect to phospholipid (Preiss et al., 1986). One known function of PI cycle diacylglycerols is to activate protein kinase C. It has been speculated that these diacylglycerols are also involved in exocytosis in that they make the relevant membranes more susceptible to fusion (Pickard & Hawthorne, 1978). We have shown that there is a liposome fusion mechanism associated with lamellar/inverted phase transitions (Ellens et al., 1989), and it is known that diacylglycerols lower lamellar/inverted phase transition temperatures (Dawson et al., 1984; Das & Rand, 1986; Epand, 1986; Coorsen & Rand, 1987). In this work, we studied the effect of 1-3 mol % diacylglycerols on the phase behavior and fusion kinetics of phospholipid systems that can form inverted phases.

Surprisingly, as little as 2 mol % diacylglycerols can lower the equilibrium lamellar/inverted hexagonal (L_α/H_{II})[1] phase transition temperature (T_H) of monomethylated dioleoylphosphatidylethanolamine (DOPE-Me)[1] by as much as 20 °C.

† This investigation was supported by Research Grant NIH-GM-31506 and UCSF Academic Senate Grant (J.B.), a senior postdoctoral grant from the California Branch of the American Cancer Society (H.E.), Grant NIH-HL-23853 (P.L.Y.), and Grant GR/E 33769 from the Science and Engineering Research Council (U.K.) (P.J.Q.).

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[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; CHOL, cholesterol; DEG, dieicosenoin; DG, diacylglycerol; DLG, dilinolenin; DOG, diolein; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOPE-Me, monomethylated dioleoylphosphatidylethanolamine; DPA, dipicolinic acid; DPG, dipalmitin; DPX, *p*-xylylenebis(pyridinium bromide); DSC, differential scanning calorimetry; NTA, nitrilotriacetic acid; OAG, 1-oleoyl-2-acetylglycerol; OArG, 1-oleoyl-2-arachidonoylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; R_0, spontaneous radius of curvature of the monolayers of a lipid–water system, as defined by Gruner (1985); T_H, lamellar/inverted hexagonal (L_α/H_{II}) phase transition temperature; ΔT_I, range of temperatures at which isotropic ^{31}P NMR resonances are observed from fully hydrated bulk multilamellar preparations of a lipid system; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

0006-2960/89/0428-3703$01.50/0

J. Am. Chem. Soc. 1993, *115*, 8096–8102

Thermotropic Properties of Model Membranes Composed of Polymerizable Lipids. 1. Phosphatidylcholines Containing Terminal Acryloyl, Methacryloyl, and Sorbyl Groups

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*Received February 5, 1993**

Abstract: The thermotropic phase behavior of hydrated bilayers of mono- and bis-substituted phosphatidylcholines (PC) containing either acryloyl, methacryloyl, or sorbyl ester groups at the chain terminus was studied by differential scanning calorimetry. Each of these compounds exhibits a single endotherm which occurs at a temperature lower than that of the main phase transition T_m of the corresponding linear saturated chain PC. Variation of the chain length of the sorbylPCs results in a pronounced odd/even alternation of the T_m. Consideration of the preferred conformation of glycerol ester lipids suggested by the crystal structure of dimyristoylPC dihydrate provides a basis for understanding the odd/even effect reported here. The interaction of the *sn*-2 chain sorbyl ester carbonyl with neighboring methylene chains appears to be predominantly intermolecular or intramolecular depending on whether the chain length is even or odd, respectively. Intermolecular interaction is expected to decrease the T_m to a greater extent than intramolecular interaction. The magnitude of the odd/even effect diminished with longer chain length as the free energy of stabilization contributed by van der Waals interchain interactions increased. A comparison of the T_m of a sorbyl ether PC and a sorbyl ester PC revealed an unexpectedly low T_m for the ether lipid. Analysis of this effect suggests previously undetected differences in the probable lipid chain conformations of ether and ester PCs. The T_m values of acryloyl-substituted PCs were somewhat higher than those of comparable chain-length sorbyl-substituted PCs. The addition of an isomethyl to the acryloyl group, i.e., methacryloyl, significantly depresses the T_m values. These systematic thermotropic studies of polymerizable lipids provide new insights into the relationship of lipid phase behavior and lipid chain substitution patterns, which is crucial to the design of novel molecules and the supramolecular assemblies formed from them.

Introduction

Hydrated supramolecular assemblies of lipids possess a variety of interesting properties, among them the ability to undergo important structural changes with temperature. These include the well known gel to liquid-crystalline phase transition of lipid bilayers as well as the transition(s) from bilayer to nonbilayer (nonlamellar) phases, e.g., inverted hexagonal (H_{II}) or inverted cubic phases (Q_{II}). These processes are highly cooperative in nature and therefore occur as well-defined temperatures. The observed thermal events result from changes in molecular packing in the nonpolar lipid chains and the polar head-group region.[1-5]

Differential scanning calorimetry (DSC) is a primary tool for the characterization of lipid bilayers.[6-9] The nonperturbing nature of DSC provides important advantages over other probe-based methods, especially for the study of lipid mixtures. The advent of high sensitivity DSC permits the direct study of lipids at sample concentrations that are comparable to those used in many liposome studies. The thermotropic transitions are observed as a maxima in the excess heat capacity vs temperature plot. The main phase transition, T_m, for the gel to liquid-crystalline transition is readily detected in addition to the total enthalpy associated with the transition ΔH_{cal}, the van't Hoff enthalpy ΔH_{vH}, and the cooperativity unit (CU) which is the ratio $\Delta H_{vH}/\Delta H_{cal}$. Small changes in lipid structure can dramatically alter these characteristics. Menger et al. reported a systematic study of bilayer perturbation by the substitution of methyl and carbonyl groups in various positions on the α- and β-chain (*sn*-1 and *sn*-2) of distearoylphosphatidylcholine (DSPC).[10-12] Both T_m and the ΔH_{cal} are diminished by chain methyl substitution, with the largest decrease occurring when the methyl group is positioned near the midpoint of the hydrocarbon chain. Lewis and McElhaney examined the effect of acyl chain length on the T_m for a variety of terminally alkyl-branched phospholipids.[13-17] The main phase transitions for methyl iso- and anteiso-branched as well as ω-*tert*-butyl- and ω-cyclohexyl-containing phosphatidylcholines (PCs) are depressed to differing extents relative to those of their unbranched analogs. Furthermore, short chain methyl iso-branched, ω-cyclohexyl-branched, and *tert*-butyl-branched PCs exhibit odd/even alternation of the T_m as the hydrocarbon chain length is sequentially increased by one carbon.[13,16,17] PCs with unbranched acyl chains do not show an odd/even alternation of the T_m.

The early 1980s saw the advent of a new class of lipid/liposome chemistry with the first reports of polymerizable lipids.[18-21] Reactive moieties—acryloyl, methacryloyl, itaconyl, dienoyl,

* Abstract published in *Advance ACS Abstracts*, August 15, 1993.

(1) Lee, A. G. *Prog. Biophys. Mol. Biol.* 1975, *29*, 3.
(2) Keough, K. M. W.; Davis, P. G. *Biomembranes* 1984, *12*, 55.
(3) Chapman, D.; Dodd, G. H. In *Structure and Function of Biological Membranes*; Rothfield, L. I., Ed.; Academic: New York, 1971; pp 31–81.
(4) Small, D. M. *Physical Chemistry of Lipids*; Plenum: New York, 1986.
(5) Jacobs, R. E.; Hudson, B.; Anderson, H. C. *Proc. Natl. Acad. Sci. U.S.A.* 1975, *72*, 3993.
(6) Mabrey, S.; Sturtevant, J. M. *Proc. Natl. Acad. Sci. U.S.A.* 1976, *73*, 3862–3866.
(7) McElhaney, R. N. *Chem. Phys. Lipids* 1982, *30*, 229–259.
(8) McElhaney, R. N. *Biochem. Biophys. Acta* 1986, *864*, 361–421.
(9) Silvius, J. R. *Chem. Phys. Lipids* 1991, *57*, 241–252.
(10) Menger, F. M.; Wood, M. G.; Zhou, Q. Z.; Hopkins, H. P.; Fumero, J. *J. Am. Chem.* 1988, *110*, 6804–6810.
(11) Menger, F. M.; Wood, M. G., Jr.; Richardson, S.; Zhou, Q.; Elrington, A. R.; Sherrod, M. J. *J. Am. Chem. Soc.* 1988, *110*, 6797–6803.
(12) Menger, F. M.; Richardson, S. D.; Wood, M. G., Jr.; Sherrod, M. *J. Langmuir* 1989, *5*, 833–838.
(13) Lewis, R. N. A. H.; McElhaney, R. N. *Biochemistry* 1985, *24*, 2431–2439.
(14) Lewis, R. N. A. H.; Sykes, B. D.; McElhaney, R. N. *Biochemistry* 1987, *26*, 4036–4044.
(15) Mantsch, H. H.; Madec, C.; Lewis, R. N. A. H.; McElhaney, R. N. *Biochemistry* 1987, *26*, 4045–4049.
(16) Lewis, R. N. A. H.; McElhaney, R. N. *Biochemistry* 1985, *24*, 4903–4911.
(17) Lewis, R. N. A. H.; Mantsch, H. H.; McElhaney, R. N. *Biophys. J.* 1989, *56*, 183–193.
(18) Regen, S. L.; Czech, B.; Singh, A. 1980, *102*, 6638–6640.

0002-7863/93/1515-8096$04.00/0

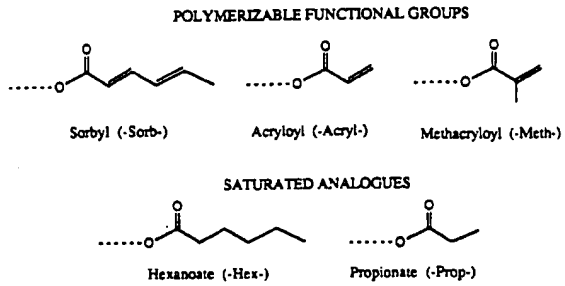


Figure 1. Structure of polymerizable and saturated esters incorporated into the ω-terminus of the acyl chain(s) of phosphatidylcholines.

muconyl, sorbyl, styryl, lipoyl, and diacetylenic—have been incorporated into lipid structures to render them polymerizable.[22,23] The reactive moiety can be positioned near the top, middle, or end of the lipid chain(s) as well as in the polar head group. The polymerization of reactive lipids in liposomes can result in significant enhancements of the liposomal chemical and colloidal stability.[22-25] Alternatively, photopolymerization of appropriately designed liposomes can be used to destabilize liposomes with leakage of aqueous contents.[26-28] An understanding of the phase behavior of bilayers composed of polymerizable lipids is crucial to the design of new molecules and the supramolecular assemblies formed from these molecules. Although most studies of polymerizable lipids include calorimetric data, which has recently been summarized,[29] there is little systematic information on the effect of the polymerizable groups on the thermotropic properties of the hydrated bilayers. In the present work, we examine the thermotropic effect of the incorporation of acryloyl, methacryloyl, and sorbyl polymerizable groups into the terminal end of the lipid hydrocarbon chain(s) of glycerol ester and ether PCs. This direct comparison of lipids synthesized and purified by the same methods and analyzed with the same instrumentation now permits new insights into the behavior of these interesting new bilayer assemblies.

The lipids characterized in this study include mono-substituted PCs, i.e., PCs with a saturated hydrocarbon *sn*-1 chain and the polymerizable group in the *sn*-2 chain, and bis-substituted PCs, which have polymerizable groups in both the *sn*-1 and *sn*-2 chains. The shorthand lipid notation is as follows: acryloyl, methacryloyl, and sorbyl functionalities are abbreviated Acryl, Meth, and Sorb, respectively, followed by the designation PC for phosphatidylcholine (Figure 1). The abbreviated name is preceded by the appropriate prefix mono or bis. The total number of atoms in each main chain minus the hydrogens is represented by a subscript at the end of the abbreviated name (note: each carbonyl is counted as one atom). The two chains may differ in length, and the subscripts represent the *sn*-1 and *sn*-2 chain lengths, respectively. Saturated (reduced) analogues of polymerizable PCs are propionate for the acryloyl esters and hexanoate for the sorbyl esters,

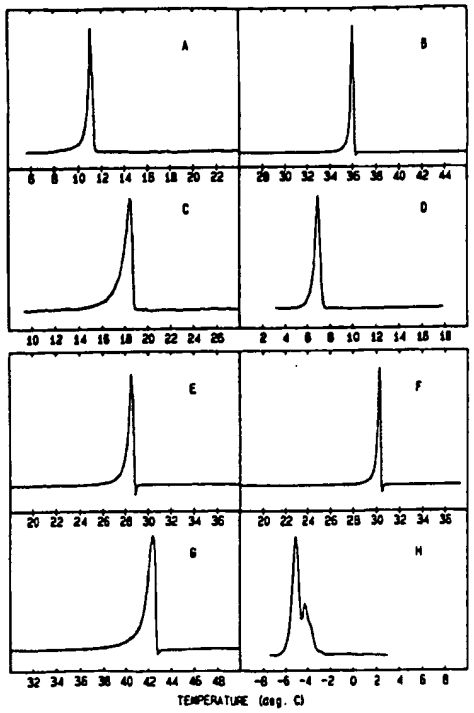
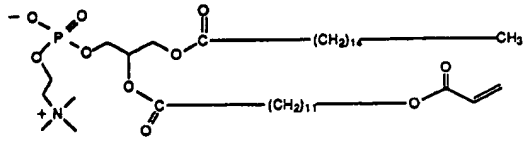


Figure 2. High-sensitivity DSC heating thermograms of aqueous dispersions of mono- and bis-SorbPC having acyl chain lengths ranging from 15 to 19 atoms as well as the saturated analog bis-HexPC$_{17,17}$. Thermograms were obtained at a scan rate between 10 and 12°/h: (A) mono-SorbPC$_{14,15}$, (B) mono-SorbPC$_{16,17}$, (C) bis-SorbPC$_{15,15}$, (D) bis-SorbPC$_{16,16}$, (E) bis-SorbPC$_{17,17}$, (F) bis-SorbPC$_{18,18}$, (G) bis-SorbPC$_{19,19}$, and (H) bis-HexPC$_{17,17}$.

which are abbreviated Prop and Hex, respectively. The structure below illustrates the notation.



mono-AcrylPC$_{16,16}$

Experimental Section

Methods. Compounds containing a UV-sensitive group were handled under yellow light. Reactions which require conditions were run under a nitrogen atmosphere. TLC was used to monitor each reaction and check the purity of the products along with ^{1}H-NMR spectra, which were obtained on a 250-MHz Bruker WM 250 spectrometer. Infrared spectra were taken on a Perkin-Elmer 983 spectrometer. UV–visible absorption spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. FAB mass spectra were obtained using an AMD modified 311A-equipped mass spectrometer with a cesium gun. The purity of the lipids was assayed by TLC both before and after storage as an amorphous ice at −10 °C in benzene. Rapid silica gel chromatography was used to purify samples prior to calorimetry.

Synthesis. Ester Lipids. A summary of the procedures described by Lamparski et al.[27] follows. Fatty acids containing polymerizable groups were prepared by monoesterification of a long chain diol followed by oxidation of the hydroxy monoester. Mono- and bis-substituted PCs containing polymerizable fatty acids were synthesized by acylation of L-α-glycerophosphatidylcholine (GPC/$CdCl_2$) or lysopalmitoylPC (lyso PC), respectively, as previously reported (Figure 2).[11,13,27,30] The crude product was purified by flash silica gel chromatography using silicic acid (Biosil-A 200–400 mesh) as the solid support with various mixtures of CH_2Cl_2/MeOH. The dried lipid powder was dissolved in benzene to a concentration between 10 and 20 mg/mL, filtered through 0.42-μm organic filters to remove trace amounts of silicic acid, bubbled with argon

(19) Hub, H. H.; Hupfer, B.; Koch, H.; Ringsdorf, H. *Angew. Chem., Int. Ed. Engl.* **1980**, *19*, 938–940.
(20) O'Brien, D. F.; Whitesides, T. H.; Klingbiel, R. T. *J. Polym. Sci., Polym. Chem. Ed.* **1981**, *19*, 95–101.
(21) Johnston, D. S.; Sanghera, S.; Pons, M.; Chapman, D. *Biochim. Biophys. Acta* **1980**, *602*, 57–69.
(22) O'Brien, D. F.; Ramaswami, V. In *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; John Wiley & Sons: New York, 1989; Chapter 17, pp 108–135.
(23) Ringsdorf, H.; Schlarb, B.; Venzmer, J. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 113–158.
(24) Regen, S. L. In *Liposomes: From Biophysics to Therapeutics*; Ostro, M. J., Ed.; Marcel Dekker: New York, 1987; p 73.
(25) Bader, H.; Dorn, K.; Hupfer, B.; Ringsdorf, H. *Adv. Polym. Sci.* **1985**, *64*, 1–64.
(26) Frankel, D. A.; Lamparski, H.; Liman, U.; O'Brien, D. *J. Am. Chem. Soc.* **1989**, *111*, 9262–9263.
(27) Lamparski, H.; Liman, U.; Barry, J. A.; Frankel, D. A.; Ramaswami, V.; Brown, M. F.; O'Brien, D. F. *Biochemistry* **1992**, *31*, 685–694.
(28) Barry, J. A.; Lamparski, H.; Shyamsunder, E.; Osterberg, F.; Cerne, J.; Brown, M. F.; O'Brien, D. F. *Biochemistry* **1992**, *31*, 10114–10120.
(29) Blume, A. *Chem. Phys. Lipids* **1991**, *57*, 253–273.
(30) Singh, A. *J. Lipid Res.* **1990**, *31*, 1522–1525.

Table I. Thermodynamic Characteristics of the Heating Endotherms of the *Mono*- and *Bis*-Sorbylphosphatidylcholines and the *Bis*-Hexanoate Analogs

PC	T_m (°C)[a]	ΔH (kcal mol^{-1})[b]	CU[c]
bis-SorbPC$_{15,15}$	18.5	9.98 ± 0.85	63 ± 2
bis-SorbPC$_{16,16}$	6.9	6.77 ± 0.14	140 ± 14
bis-SorbPC$_{17,17}$	28.8	7.48 ± 0.16	167 ± 9
bis-SorbPC$_{18,18}$	30.3	10.83 ± 0.14	210 ± 6
bis-SorbPC$_{19,19}$	42.5	10.83 ± 0.14	75 ± 7
mono-SorbPC$_{14,15}$	11.0	6.99 ± 0.62	166 ± 32
mono-SorbPC$_{16,17}$	36.1	10.5 ± 0.29	202 ± 22
bis-HexPC$_{17,17}$[d]	−5.0 (−7.5)[e]	10.7 ± 0.2[e]	nd[f]
bis-Sorb ether PC$_{17,17}$	11.4	8.5 ± 0.1	nd
bis-Hex ether PC$_{17,17}$[d]	−15.4 ± 1.1[e]	10.7 ± 0.2[e]	nd

[a] The T_m values varied by less than ±0.1° between successive runs, except for *bis*-Hex ether PC$_{17,17}$. [b] The ΔH values quoted are the average of the total enthalpy for succesive runs. [c] The CU values are the average for successive runs. [d] Samples exhibiting a T_m below 0 °C were hydrated in aqueous ethylene glycol: 35% ethylene glycol/aqueous buffer for Microcal MC-2 DSC and 44% ethylene glycol/aqueous buffer for Perkin-Elmer DSC-7. [e] Measured by Perkin-Elmer DSC-7. [f] nd = not determined.

for 10 min, and then stored as an amorphous ice at −10 °C. Overall yields ranged from 50 to 85% on the basis GPC-$CdCl_2$ or lysoPC.

Sorbyl Ether Glycerophosphocholine. The synthesis of the ether lipids were described by Lee and O'Brien.[31]

UV Polymerization of Mono-SorbPC. Extended bilayers of mono-SorbPC$_{14,15}$ were prepared in a manner identical to that described for DSC samples. The lipid suspension was placed in a 3-mL quartz cuvette and photopolymerized for 75 min by exposure to 254-nm light from a low-pressure Hg lamp located 1 cm away. The sample was agitated by bubbling argon. The loss of monomeric mono-SorbPC$_{14,15}$ was monitored by the decrease in the sorbyl chromophore at 256 nm.[27,32]

Differential Scanning Calorimetry. The lipids were lyophilized from benzene to a floculent powder and hydrated to a concentration of either 1 or 1.5 mg/mL with degassed buffer (10 mM Na_2HPO_4/150 mM NaCl at pH 7.4). Concentrations were determined by accurately weighing out known amounts of lipid (typically 5–10 mg) and adding the appropriate volume of buffer. Lipid suspensions were vortexed at temperatures greater than the T_m for 3–5 min until uniformity was observed followed by 10 freeze-thaw cycles (isopropyl alcohol/dry ice temperature to room temperature). High-sensitivity calorimetric thermograms were obtained on aqueous buffer aliquots (1.2631-mL cell volume) of lipids having endotherms at temperatures above 0 °C with a Microcal Model MC-2 differential scanning calorimeter. Thermograms were obtained at temperatures down to −7.0 °C from samples dispersed in a 35% ethylene glycol/aqueous buffer mixture. A scan rate between 10° and 15°/h was employed for all high-sensitivity measurements. A Perkin-Elmer DSC-7 differential scanning calorimeter was used to characterize the behavior of hydrated lipids (44% ethylene glycol/water) with endotherms below −6.0 °C.

Results

DSC heating curves were obtained on each of the polymerizable PCs and their saturated analogues. The thermodynamic parameters reported are the average of two different samples, each of which was scanned three times. The main phase transition temperature T_m, calorimetric enthalpy ΔH, and cooperative unit (number of lipids undergoing the phase transition at the same time; determined by dividing the van't Hoff enthalpy by the calorimetric enthalpy) are reported in Tables I and II.

SorbylPCs. Aqueous dispersions of five bis-substituted and two mono-substituted SorbPCs of different chain lengths were studied (Table I). Representative endotherms are shown in Figure 2. Each SorbPC lipid exhibited a single sharp transition. Pretransitions were not observed in any of these samples. In some cases, repetitive calorimetric scans produced thermograms, which were progressively broader with slightly depressed T_ms and smaller lipid cooperativity. This unusual behavior could possibly be due to low conversions of lipid to polymer or lipid hydrolysis to form small amounts of fatty acids during the heating scans to 65 °C.[13] However, in our experience, the SorbPCs do not polymerize on heating in the absence of initiator. Low levels of impurities (<1%) which are not readily detected by TLC or ^{1}H NMR will cause broadening of the main transition with a decrease in the lipid cooperative unit.[13] Samples of both bis-SorbPC$_{15,15}$ and bis-SorbPC$_{19,19}$ exhibited broad main transition endotherms indicating poor lipid cooperativity. This broadening of the endotherm could be either a function of the lipid-phase behavior or due to the presence of undetected impurities. Greater lipid cooperativity was observed for the hydrated bilayers of bis-SorbPC$_{16,16}$, bis-SorbPC$_{17,17}$, and bis-SorbPC$_{18,18}$.

The T_m at 28.8 °C observed by DSC for bis-SorbPC$_{17,17}$ was substantiated by X-ray diffraction and ^{31}P-NMR spectrometry. The X-ray diffraction revealed a transition from a well-ordered gel state to a disordered liquid-crystalline state as the sample was thermally cycled from temperatures below to above 29 °C.[33] The ^{31}P-NMR spectrum of the sample in the same temperature region showed a decrease in the basal line width above the T_m, as expected for the increased motion and the axial symmetry of the phosphate head group on the ^{31}P-NMR time scale.[34]

The melting temperatures of the sorbyl alcohols and fatty acids as well as the T_ms of the bis-SorbPCs are plotted as a function of acyl chain length in Figures 3 and 4, respectively. The sorbyl alcohol shows a distinctive odd/even alternation of the melting point with increasing hydrocarbon chain length. This means that the melting point of alcohols with an even number chain length

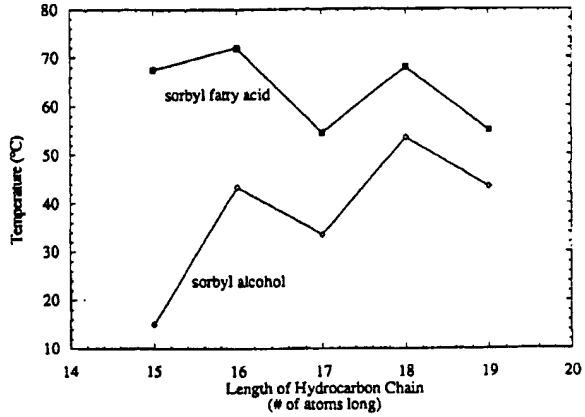


Figure 3. Chain-length dependence of the melting point of long chain sorbyl alcohols and fatty acids.

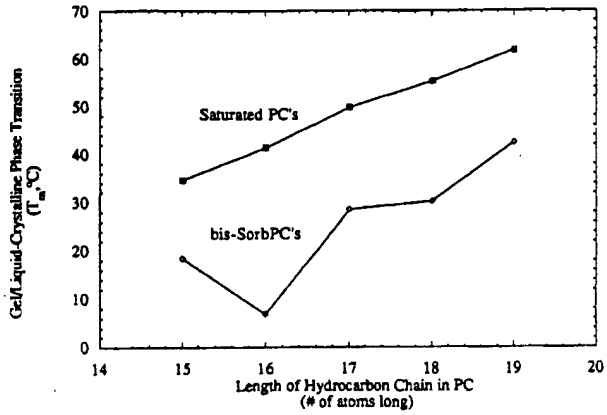


Figure 4. Chain-length dependence of the gel/liquid-crystalline phase transition temperature T_m of bis-SorbPCs.

(31) Lee, Y.-S.; O'Brien, D. F. *Chem. Phys. Lipids* **1992**, *61*, 209–218.
(32) Tyminski, P. N.; Ponticello, I. S.; O'Brien, D. F. *J. Am. Chem. Soc.* **1987**, *109*, 6541–6542.
(33) Shyamsunder, E.; Lamparski, H.; O'Brien, D. F., unpublished observations.
(34) Seelig, J. *Biochim. Biophys. Acta* **1978**, *515*, 105–140.

is greater than the average of the melting points of the two nearest odd number chain-length alcohols. The melting-point behavior of the sorbyl fatty acids shows a similar odd/even alternation. Odd/even melting-point behavior is characteristic of saturated alkanes[35] as well as some alkyl-substituted fatty acids.[13,14,16,17] The T_ms of the bis-SorbPCs are also dependent on the lipid chain length in an alternating even/odd manner. Figure 4 shows that the T_m of lipids with even-numbered chains is lower than the average T_m of the two adjacent odd-numbered chain lipids in the series. In the case of the three lipids with chain lengths of 15, 16, and 17, bis-SorbPC$_{16,16}$ has a lower T_m than each of the other two. Comparison of lipids with chain lengths of 17, 18, and 19 shows that the T_m for bis-SorbPC$_{18,18}$ is lower than the average of the other two but still greater than that of bis-SorbPC$_{17,17}$. These data indicate that the apparent odd/even effect is attenuated as the chain length is increased.

A comparison of T_m values for bis-SorbPCs and the corresponding saturated chain acylPCs of the same chain length reveals a notable depression in T_ms for all of the SorbPCs (Figure 4). This effect is especially pronounced when lipids of even number chain length are compared, e.g., bis-SorbPC$_{16,16}$ has a T_m of 6.9 °C compared to 41.4 °C for dipalmitoylphosphatidylcholine (DPPC, PC$_{16,16}$), because the unbranched saturated PCs do not exhibit an odd/even alternation in T_m values.

Two mono-substituted SorbPCs were examined, and each showed a single endotherm (Table I), as was the case for the bis-SorbPCs, which presumably corresponds to the gel/liquid-crystalline phase transition. The longer chain mono-SorbPC$_{16,17}$ (*sn*-1, palmitoyl chain; *sn*-2, sorbyl fatty acid, 17 atoms in length) showed a higher T_m, enthalpy, and cooperative unit than the shorter mono-SorbPC$_{14,15}$. Comparison of the T_m for the mono-SorbPCs to that of unbranched saturated PCs is complicated by the unequal length of the *sn*-1 and *sn*-2 chains in the mono-SorbPCs. An estimate of the expected T_m for a PC with a 14-carbon *sn*-1 chain and a 15-carbon *sn*-2 chain can be obtained by averaging the values for PC$_{14,14}$ (DMPC) and PC$_{14,16}$, which are 23.6 and 35 °C,[36] respectively. This suggests that the T_m of PC$_{14,15}$ should be about 29 °C. A similar analysis for PC$_{16,17}$ predicts the T_m value to be near 45 °C (based on the reported T_m of 41.4 °C for PC$_{16,16}$ and 49 °C for PC$_{18,18}$[36]). These estimated values indicate that the T_m of the shorter chain mono-SorbPC$_{14,15}$ is depressed by 18° whereas the incorporation of the reactive sorbyl ester into the longer chain mono-SorbPC$_{16,17}$ reduces its T_m by 9° from that expected for a saturated PC$_{16,17}$. The apparent effect of chain length on the magnitude of the perturbation of T_m will be considered further in the Discussion.

The T_m of extended bilayers of mono-SorbPC$_{14,15}$ was lost upon photopolymerization of the lipid. The loss of this thermotropic transition suggests entanglement and/or a steric arrangement of the linear polymer chains that are formed during the photopolymerization. Further consideration of the effect of polymerization on the phase behavior of the hydrated assemblies will be addressed in a subsequent study.

Acryloyl- and MethacryloylPCs. DSC thermograms of mono- and bis-acryloyl- and methacryloylPCs are depicted in Figure 5 (Table II). Both the mono- and bis-substituted PCs have the same chain length as PC$_{16,16}$ (T_m 41.4 °C), i.e., acryloyl and methacryloyl fatty acids are 16 atoms long. The incorporation of a single acryloyl group into the terminus of the *sn*-2 chain of mono-AcrylPC$_{16,16}$ decreases the T_m to 31.8 °C. The substitution of a second acryloyl group into the other chain, bis-AcrylPC$_{16,16}$, caused a further decrease of the T_m by only another 2 °C. Thus, the effects of the first and second acryloyl groups are not additive. Methacryloyl substitution caused an even greater perturbation of the bilayer, e.g., the T_m of mono-MethPC$_{16,16}$, is almost 30° lower than that of PC$_{16,16}$. The lipid with two methacryloyl groups, bis-MethPC$_{16,16}$, has a 15° lower T_m. Again, the second polymerizable ester group does not have as great an effect on the T_m as the first substitution.

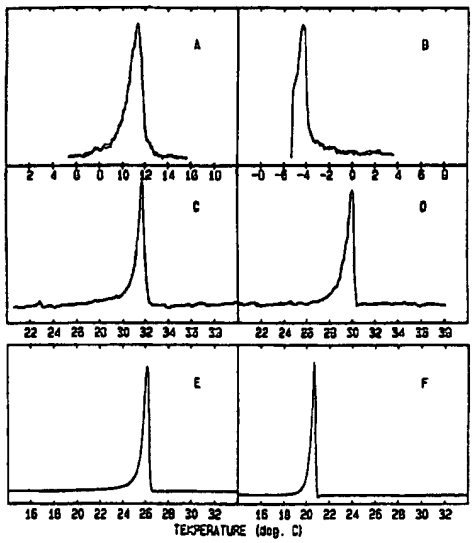


Figure 5. High-sensitivity DSC heating thermograms of aqueous dispersions of mono- and bis-acryloyl- and methacryloylPC's and the saturated analogs mono- and bis-propionate PC. The total chain length of the PCs is 16 atoms long. Thermograms were obtained at a scan rate between 10 and 12°/h: (A) mono-MethPC$_{16,16}$, (B) bis-MethPC$_{16,16}$, (C) mono-AcrylPC$_{16,16}$, (D) bis-AcrylPC$_{16,16}$, (E) mono-PropPC$_{16,16}$, and (F) bis-PropPC$_{16,16}$.

Table II. Thermodynamic Characteristics of the Heating Endotherms of the *Mono*- and *Bis*-Acryloyl- and Methacryloylphosphatidylcholines and the Saturated *Mono*- and *Bis*-Propionate Analogs

PC	T_m (°C)[a]	ΔH (kcal mol^{-1})[b]	CU[c]
mono-AcrylPC$_{16,16}$	31.8	8.90 ± 0.42	72 ± 10
bis-AcrylPC$_{16,16}$	30.0	6.90 ± 0.59	118 ± 20
mono-MethPC$_{16,16}$	11.4	10.50 ± 0.25	38 ± 0
bis-MethPC$_{16,16}$[d]	−4.4	5.30 ± 0.26	96 ± 6
mono-PropPC$_{16,16}$	26.1	12.77 ± 0.85	71 ± 4
bis-PropPC$_{16,16}$	20.7	12.31 ± 1.01	115 ± 14

[a] The T_m values varied by less than ±0.1° between successive runs. [b] The ΔH values quoted are the average of the total enthalpy for successive runs. [c] The CU values are the average for successive runs. [d] Sample was hydrated in 35% ethylene glycol/aqueous buffer.

Both the calorimetric enthalpy and cooperativity of the phase transition are affected by the incorporation of acryloyl and methacryloyl groups into the PC. The addition of a second acryloyl group or methacryloyl group lowers the transition enthalpy by 2 and 5 kcal/mol, respectively. The lipid cooperativity was increased slightly by the acryloyl substitution and was doubled for the methacryloyl lipids. Clearly, both the carbonyl and methyl groups cause significant perturbations of the lipid bilayer packing.

Saturated Ester Models for Acryloyl- and SorbylPCs. Saturated ester-substituted lipids, which are identical in acyl chain length and carbonyl location as mono- and bis-AcrylPC$_{16,16}$ as well as bis-SorbPC$_{17,17}$, were prepared as model lipids to distinguish between the effect of the ester chain substitution and the presence of double bond(s) in the lipid tail on the lipid phase behavior (Figures 2 and 5). Each of these saturated ester PCs exhibited a single main transition (Tables I and II) with even lower T_m values than the corresponding AcrylPC and SorbPC lipids. The T_m of mono-PropPC$_{16,16}$ at 26.1 °C is 5.7° lower than that of mono-AcrylPC$_{16,16}$, and the T_m of bis-PropPC$_{16,16}$ is 20.7 °C, which is 9.3° below that of bis-AcrylPC$_{16,16}$. The effects of the saturated ester groups are nearly additive. A greater effect was observed for bis-HexPC$_{17,17}$, which showed a T_m at −5.0 °C (−7.5 °C when measured by low-sensitivity DSC). This was a 32°

(35) Broadhurst, M. G. *J. Res. Natl. Bur. Stand., Sect. A* **1962**, *A66*, 241–249.
(36) Chen, S. C.; Sturtevant, J. M. *Biochemistry* **1981**, *20*, 713–718.

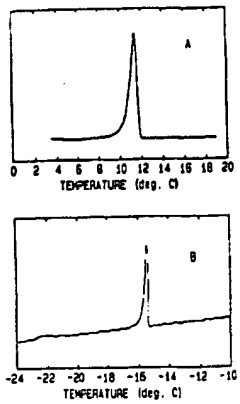
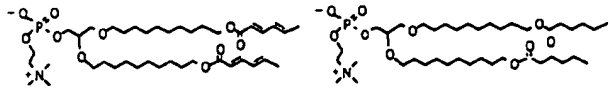


Figure 6. DSC heating thermograms of bis-ether PCs: (A) high-sensitivity DSC of bis-Sorb ether $PC_{17,17}$, scan rate of 10–12°/h; (B) low-sensitivity DSC of bis-Hex ether $PC_{17,17}$ (14.9 wt % in 44% ethylene glycol/aqueous buffer), scan rate of 1°/min.

decrease in the T_m compared to that of parent bis-Sorb$PC_{17,17}$ and a 54° decrease compared to that of the completely saturated dimargonylPC ($PC_{17,17}$, T_m = 49 °C). The difference in the T_m between bis-Prop$PC_{16,16}$ and $PC_{16,16}$ was 21°. In the latter case, the carbonyl group is closer to the lipid chain terminus.

Ether PCs. The two ether lipids shown were studied in order to compare the thermotropic behavior of chain-substituted ether and ester PCs (Table I, Figure 6). Both bis-Sorb ether $PC_{17,17}$ and its saturated analog, bis-Hex ether $PC_{17,17}$, were prepared as described previously.[31] The T_m of bis-Sorb ether $PC_{17,17}$ was



observed at 11.4 °C, which can be compared to the value of 28.8 °C for the corresponding ester PC. The significantly lower phase-transition temperature for the ether lipid was unexpected because ether PCs generally exhibit a somewhat higher T_m than the corresponding ester PC of the same chain length, e.g., the T_ms of $PC_{16,16}$ (DPPC) and 1,2-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC) are 41.4 and 43.7 °C, respectively. The ability of ether lipids to pack more closely together than ester lipids, due to the absence of the carbonyl groups, has been proposed to account for the somewhat higher T_m.[37,38] Possible reasons for the exceptional departure of bis-Sorb ether $PC_{17,17}$ from the usual behavior will be discussed below. Bis-Hex ether $PC_{17,17}$ showed a T_m at −16.2 °C, which is about 27° below that of the polymerizable bis-Sorb ether $PC_{17,17}$. The magnitude of the effect is similar to that found for bis-Hex$PC_{17,17}$ (see above).

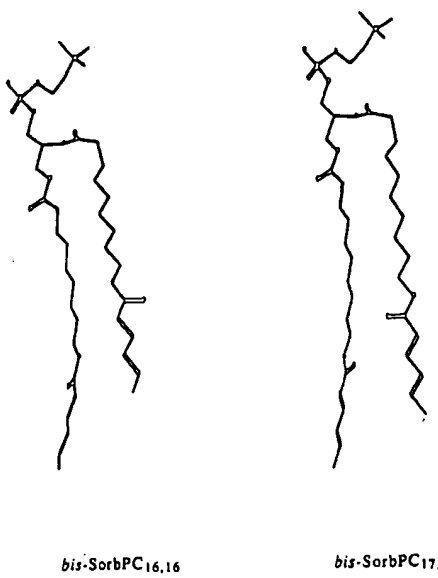
Discussion

The thermotropic effects caused by the incorporation of polymerizable substituents into the terminal ends of the lipid chains are consistent with previous studies of branched chain lipids and reveal important aspects of these hydrated lipid assemblies. In all cases, the incorporation of an ester functionality, whether containing a polymerizable moiety or its saturated analog, into the hydrocarbon chain resulted in a depression of the main phase transition, T_m, compared to that of saturated unbranched PCs of similar chain length. These results are consistent with the previous studies of Lewis and McElhaney on the methyl iso- and anteiso-branched and ω-cyclohexyl- and ω-*tert*-butyl-substituted PCs.[13-17] Methyl substituents at various positions on the *sn*-2 chain of the PCs decrease the T_m to different extents depending upon the location of the methyl group on the chain.[10] In order to account for the decreases in both the T_m and the enthalpy, it is necessary to examine the energetics of the transition process. The gel phase is characterized by fully extended hydrocarbon chains in the all-trans conformation. At the phase transition, there is a reduction in van der Waals interchain interactions and an increase in the number of gauche C–C linkages. Schindler and Seelig calculated a value of 4.3 gauche conformers/chain on the basis of ^{2}H-NMR order parameters.[39] Recent FT–IR studies reported that there are between 3 and 4.2 gauche conformers/chain in deuterated-DPPC analogs[40] and 3.7 gauche conformers/chain in DPPC.[41] This results in an increase in the molecular area by 6 Å^2/chain and a decrease in the bilayer thickness by 20%.[42] Nagle has shown that van der Waals interchain interactions contribute 4.1, 5.5, and 7.3 kcal/mol to the transition enthalpy for $PC_{14,14}$ (DMPC), $PC_{16,16}$ (DPPC), and $PC_{18,18}$ (DSPC), respectively.[42,43] The introduction of a methyl group or other branching substituent reduces the attractive van der Waals interactions and significantly decreases the T_m and enthalpy of transition. McFarland and McConnell suggested that the lipid chains are endowed with a critical bend near their center which divides the chains into two segments of comparable length.[44] The segment associated with the head group lies at a 30° angle from the membrane surface, while the lower segment lies perpendicular to the surface. Substituents near the center of the chain stabilize the critical bend that develops when the gel phase reorganizes into a liquid-crystalline phase, thereby appreciably lowering the T_m and the ΔH_{cal} relative to those of an unbranched lipid. Since the chain segment between the central bend and the terminal methyl permits the segments to rotate along the locus of a cone with the bend at its apex, there is more interaction space available near the chain terminus to accommodate branching or kinking. Therefore, a substituent near the end of the chain causes far less packing distortion than a midchain substitution. The effects observed in this study of polymerizable PCs will be considered in light of these previously reported trends.

One of the main features of the present results is the pronounced odd/even alternation in the T_m observed for the bis-SorbPCs (Figure 4). Somewhat similar behavior was reported for ω-cyclohexyl, ω-*tert*-butyl, and short chain methyl iso-branched PCs.[13,16,17] Lewis and McElhaney reported that the odd/even effect is enhanced in compounds that contain bulky substituents such as a *tert*-butyl or a cyclohexyl at the ω-position and a polar component at the α-position.[16,17] While the sorbyl moiety is not as bulky as a cyclohexane ring, the protruding ester carbonyl and the relative stiffness of the trans diene appear to be sufficient to alter the packing in the crystal lattice between odd- and even-length chains. Odd/even discontinuities are also often observed in the solid-state behavior of several long chain paraffins as well as in the melting points of a variety of fatty acids.[13,17,35,45,46] The odd and even chain-length sorbyl alcohols and fatty acids also exhibit alternating melting points with chain length (Figure 4). The observed solid-state phenomenon results from different end-group interactions which, in turn, effect the tilting of odd- and even-length chains during formation of the crystalline lattice.

Consideration of the preferred conformation of glycerol ester lipids suggested by the crystal structure of dimyristoylPC ($PC_{14,14}$) dihydrate[47] provides a basis for understanding the odd/even alternation observed in certain substituted PCs. Groups attached near the ω-terminus of the *sn*-1 and/or *sn*-2 acyl chain of a PC

(37) Smaby, J. M.; Hermetter, A.; Schmid, P. C.; Paltauf, F.; Brockman, H. L. *Biochemistry* **1983**, *22*, 5808–5813.
(38) Hing, F. S.; Maulik, P. R.; Shipley, G. G. *Biochemistry* **1991**, *30*, 9007–9015.
(39) Schindler, H.; Seelig, J. *Biochemistry* **1975**, *14*, 2283–2287.
(40) Mendelsohn, R.; Davies, M. A.; Brauner, J. W.; Schuster, H. F.; Dluhy, R. A. *Biochemistry* **1989**, *28*, 8934–8939.
(41) Casal, H.; McElhaney, R. N. *Biochemistry* **1990**, *29*, 5423–5427.
(42) Nagle, J. F. *Ann. Rev. Phys. Chem.* **1980**, *31*, 157–195.
(43) Nagle, J. F.; Wilkinson, D. A. *Biophys. J.* **1978**, *23*, 159–175.
(44) McFarland, B. G.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1274.
(45) Ishizawa, A. *Nippon Kagaku Zasshi* **1971**, *89*, 516.
(46) Ishizawa, A. *Nippon Kagaku Zasshi* **1971**, *89*, 815.
(47) Pearson, R. H.; Pascher, I. *Nature* **1979**, *281*, 499–501.



bis-SorbPC$_{16,16}$ *bis*-SorbPC$_{17,17}$

Figure 7. Drawings of the all-trans extended conformation of bis-SorbPC$_{16,16}$ (left) and bis-SorbPC$_{17,17}$ (right).

are inequivalent to one another since the *sn*-1 chain penetrates further into the bilayer. If the SorbPC acyl chains have a similar orientation as those of PC$_{14,14}$, then the mode of interaction between substituents on the *sn*-1 and *sn*-2 chains will depend on whether there are an odd or even number of atoms in the chain. Figure 7 shows the conformation of bis-SorbPC$_{16,16}$ and bis-SorbPC$_{17,17}$, respectively, which were derived from the structure of PC$_{14,14}$[47,48] by extending and modifying the two acyl chains to incorporate the terminal sorbyl ester groups. In each drawing, the sorbyl ester carbonyl on the *sn*-1 chain points toward a neighboring lipid which would be located either in front of the plane of the page or behind the plane of the page. The energetic contribution of this carbonyl should be similar in each compound. The sorbyl ester carbonyl on the *sn*-2 chain of these lipids lies in the plane of the page. In the case of the even-numbered chain bis-SorbPC$_{16,16}$, the carbonyl oxygen points toward a neighboring lipid chain, whereas in the example of an odd-numbered chain lipid (bis-SorbPC$_{17,17}$), the carbonyl oxygen points toward the second chain of the same lipid. The carbonyl–methylene interaction of the *sn*-2 chain sorbyl ester carbonyl appears to be predominantly intermolecular or intramolecular depending on whether the chain length is even or odd, respectively. Energetic differences between intramolecular and intermolecular interactions are expected to exist regardless of the lipid chain tilt to the bilayer normal. Therefore, PCs with either even- or odd-numbered chains may have similar crystallographic structures yet differ in their energetics. A similar explanation was proposed for the observed odd/even alternation exhibited by ω-*tert*-butylPCs,[17] whereas differences in acyl chain tilt were proposed to account for the odd/even behavior in ω-cyclohexyl[17] and short chain methyl iso-branched PCs.[13] Note that the odd/even effects observed for short chain methyl iso-branched PCs can also be explained by intra- or intermolecular interactions between methyl groups and nearest neighbor methylenes, e.g., methyl iso-branched PCs of odd-numbered chain lengths have their methyl groups directed inward in the same manner as the carbonyl of odd-length SorbPCs. Both types of substituents influence the T_m in a similar fashion. In other words, PCs with either alkyl or carbonyl substituents oriented in a manner that gives preferential intramolecular interaction have higher T_m values than PCs where the orientation of the substituents favors intermolecular interaction. In the latter case, the substituents perturb the bilayer to a greater extent with a consequent destabilization of the gel phase and stabilization of the liquid-crystalline phase. The increased molecular area per molecule due to the formation of kinks in the lipid chain at temperatures above the T_m will be stabilized by substituents which point away from the lipid. On the other hand, substituents directed toward the other chain of the same lipid will have a smaller stabilizing effect on the liquid-crystalline phase.

The significant difference in thermotropic behavior between bis-SorbPC$_{15,15}$ and bis-SorbPC$_{16,16}$ also signals the presence of a more stable gel phase in the former lipid. The increase in gel-state stability is indicated by the transition entropy ΔS, which was 34 cal mol^{-1} K^{-1} for bis-SorbPC$_{15,15}$ and 24 cal mol^{-1} K^{-1} for bis-SorbPC$_{16,16}$. The studies of McElhaney and co-workers[13,16,17] indicate that PCs with branched terminal groups can form different types of gel phases depending on whether the chain has an even or odd number of atoms. Although we do not presently have spectroscopic evidence for the existence of different types of gel phases for the SorbPCs, this possibility will be explored in the future.

It can be seen in Figure 4 and Table I that the decrease in T_m for the longer even chain-length bis-SorbPC$_{18,18}$ is less than that of the shorter chain bis-SorbPC$_{16,16}$. If the phase transition temperature of all bis-SorbPCs where linearly dependent on acyl chain length over the range investigated, the even-length PCs of 16 and 18 atoms would have predicted T_ms of 23.2 and 35.7 °C (computed from the midpoint of adjacent odd-length SorbPCs). However, the difference between the predicted and experimental T_ms were 16.3° and 5.4°, respectively. This is probably a consequence of the free energy stabilization contributed by van der Waals interchain interactions, which increase with the acyl chain length. Eventually, the van der Waals interactions will be sufficiently greater than the energy associated with the steric and dipolar effects of the carbonyl oxygen that the van der Waals interactions will dominate bilayer stabilization. The odd/even discontinuities in T_m observed in Figure 4 should eventually disappear for longer acyl chains.

The phase behavior observed for both mono-substituted SorbPCs, mono-SorbPC$_{14,15}$ and mono-SorbPC$_{16,17}$, resembles that of the corresponding odd chain-length bis-substituted PCs because the sorbyl ester carbonyl on the *sn*-2 chain is oriented to primarily favor intramolecular interactions. Comparison of the T_m values for these two lipids to those of the corresponding unbranched saturated chain PCs indicates that the ester carbonyl in mono-SorbPC$_{14,15}$ reduces the T_m by 18° and in the mono-SorbPC$_{16,17}$, the T_m is reduced by only 9°. These data may be interpreted in the same manner as proposed for the bis-SorbPCs, that is, the van der Waals chain interactions become relatively more important as the chain length increases and the ester carbonyl is displaced further from the lipid backbone.

The second focus of this study involves the mono- and bis-substituted PCs which contain either acryloyl or methacryloyl substituents (Table II) in the hydrocarbon chain terminus. It is helpful to recall the effect of simple methyl substitution at various positions of the *sn*-2 chain of distearoylPC (PC$_{18,18}$).[10] Methyl substitution at carbons 4, 10, or 16 of PC$_{18,18}$ caused a decrease in the T_m from 54.8 to 41.5, 5.6, and 38.5 °C, respectively. A parallel decrease in transition enthalpy was observed as the methyl substituent position was varied along the hydrocarbon chain. Note that in each of these cases, an even-numbered carbon was the site of methyl substitution, which favors intramolecular interaction. The ester carbonyl of both acrylyol and methacryloyl PCs is located in the same position on the acyl chain as the methyl group of methyl anteisoDPPCs described by Lewis and McElhaney.[14] The ester carbonyl of mono-AcrylPC$_{16,16}$ decreases the T_m by about 11 °C from that of PC$_{16,16}$, whereas the methyl in the equivalent-length methyl anteisoPC decreases the T_m by 32 °C. This appears to be a consequence of the smaller volume occupied by the carbonyl compared to a methyl substituent which rotates about the C–C bond. The T_m of mono-MethPC is much

(48) Vanderkooi, G. *Biochemistry* **1992**, *30*, 10760–10768.

lower than that of mono-AcrylPC, since the methacrylyol group contains both a carbonyl in the anteiso position and a methyl group in the iso position, thereby occupying an even greater volume in the chain terminus which decreases the van der Waals attraction forces.

Comparison of the T_ms of bis-Acryl$PC_{16,16}$ (30.0 °C) and bis-Sorb$PC_{16,16}$ (6.9 °C) illustrates the combined effect of the location of the ester group along the 16-atom chain and the orientation of the terminal ester carbonyl in a manner that favors either intermolecular or intramolecular interactions. In bis-Acryl$PC_{16,16}$, the ester carbonyl is located at the C-14 position of the acyl chain, which, as noted earlier, causes a 11° decrease in the T_m from that of the reference $PC_{16,16}$ (DPPC). The *sn*-2 ester carbonyl at this chain position is expected to primarily interact intramolecularly with the neighboring acyl chain of the same lipid. In contrast, the bis-Sorb$PC_{16,16}$ ester carbonyl is located at the C-11 position of the acyl chain where it is closer to the critical bend at the chain midpoint. The decrease in T_m is consistent with the trends expected from the previous methyl substitution studies. However, the effect is magnified by the orientation of the *sn*-2 sorbyl ester carbonyl at the odd-atom chain position, which favors intermolecular carbonyl–methylene interaction leading to a greater decrease in T_m.

In a preliminary manner, we have examined how the lipid T_m is influenced by the unsaturation of the polymerizable moieties located in the acyl terminus. The saturated analogs of mono- and bis-Acryl$PC_{16,16}$ and bis-Sorb$PC_{17,17}$ exhibit significantly lower T_ms than the parent polymerizable lipids. This effect appears unusual when considering that unsaturation in the acyl chain reduces the van der Waals attraction forces due to poorer bilayer packing and effectively lowers the enthalpic parameters for melting. However, trans olefins and dienes cause less disruption of the bilayer packing than the corresponding cis double bonds. Furthermore, the effect of unsaturation on chain packing is decreased as the unsaturation is moved down the chain away from the critical bend in the same manner as observed for methyl-branched PCs.[10] The observed T_ms indicate that whereas the ester carbonyls in these lipids destabilize the gel phase, the "ene" in AcrylPC and the "diene" in SorbPC may provide some stabilization of the gel phase, perhaps by π–π-overlap of the double bond(s) with adjacent PCs. Saturation of these groups increases the number of C–C gauche conformations, allows greater freedom of rotation of the C–C σ-bonds compared to C–C π-bonds, and eliminates any π–π-overlap. Each of these effects serves to destablize the gel phase and depress the T_m. The observed decrease in T_m for saturated Prop$PC_{16,16}$ compared to Acryl$PC_{16,16}$ is probably due to the greater motion of the C–C σ-bond as well as to the loss of the π–π-overlap of orbitals. An increase in the number of gauche conformers is unlikely since only one C–C σ-bond is added to each acyl chain. The even larger decrease in T_m exhibited by Hex$PC_{17,17}$ compared to Sorb$PC_{17,17}$ could be due to a combination of all three factors affecting the packing of the chain.

The distinctive thermotropic characteristics of bis-Sorb ether $PC_{17,17}$ was initially puzzling. Ether PCs usually exhibit a slightly higher T_m than that of the same chain-length ester PC. However, in the odd-atom chain-length bis-Sorb ether $PC_{17,17}$, the T_m was more than 17° lower than that of bis-Sorb$PC_{17,17}$. An interesting hypothesis to account for this seeming anomaly is an extension of the previous consideration of the orientation of the carbonyl oxygen in the all-trans extended conformation of the chain. The sorbyl ester carbonyl in the *sn*-2 chain of bis-Sorb$PC_{17,17}$ is expected to primarily interact in an intramolecular manner as discussed earlier. The conformations of the ester groups which connect the acyl chain to the glycerol backbone are shown in Figure 7. The carbonyl of the *sn*-2 chain in ester PCs is directed toward the water-bilayer interface,[47] where it may hydrogen bond with water.[49] The structure of fluid PC bilayers determined by the joint refinement of X-ray and neutron diffraction data indicates that water permeates into and beyond the region of the glycerol backbone in dioleolylPC.[50] Therefore, H-bonding is likely to contribute to the overall energetics which control the preferred conformation of the *sn*-2 PC chain. When the lipid structure is changed from an ester PC (bis-Sorb$PC_{17,17}$) to an ether PC (bis-Sorb ether $PC_{17,17}$), the ester carbonyl at the glycerol backbone becomes a methylene group which no longer hydrogen bonds. We suggest that the absence of H-bonding permits the *sn*-2 ether PC chain to adopt a different conformation than that shown in Figure 7 for the ester PC. Any change in the orientation of the chain near the glycerol backbone will alter the orientation of the carbonyl at the 12-position and significantly increase the probability of sorbyl ester carbonyl interaction with neighboring lipids. To the extent that intermolecular interaction is increased, the T_m will be depressed. The absence of comparative crystallographic or modeling data for the glycero ether and glycero ester lipids limits further analysis of the lipid chain conformations. The unusual nature of the comparative calorimetric data reported here is so striking that future crystallographic and modeling studies of ether and ester lipids should be pursued to more fully characterize the differences between these lipid classes, e.g., these calorimetric differences may be due to the presence of different gel phases in the ester and ether PCs.

This report describes the first systematic study of the thermotropic behavior of chain-substituted polymerizable lipids. These results provide new insights into the effect of molecular substitution patterns on supramolecular-phase behavior. The lipid gel to liquid-crystalline phase transition T_m is sensitive to both the location of the functional group along the chain as well as to the orientation of branching substituents, e.g., terminal ester carbonyls. The observed odd/even effect highlights the probable consequence of chain orientation in ester lipids. Furthermore, the usual behavior of Sorb ether $PC_{17,17}$ suggests previously unsuspected differences in the chain packing of ether and ester PCs. These current and future studies will facilitate the design of novel lipids and materials on the basis of supramolecular assemblies of those lipids.

Acknowledgment. The authors thank the National Science Foundation for support of this research. We thank Dr. Nathan Collins for assistance in preparing the structures shown in Figure 7. We also thank Dr. E. Shyamsunder, Princeton University, for X-ray diffraction characterization of bis-Sorb$PC_{17,17}$.

(49) Gawrisch, K.; Ruston, D.; Zimmerberg, J.; Parsegian, V. A.; Rand, R. P.; Fuller, N. *Biophys. J.* **1992**, *61*, 1213–1223.
(50) Wiener, M. C.; White, S. H. *Biophys. J.* **1992**, *61*, 434–447.